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22. EFFECT OF DIPHTHERIA TOXIN UPON TISSUE ENZYMES *IN VITRO*

By BENJAMIN ALFRED PETERS and ROBERT NEIL CUNNINGHAM

From Ham Green Hospital, Bristol

(*Received 25 October 1940*)

The normal course of diphtheria is generally characterized by pronounced ketonuria and other symptoms indicating serious metabolic disturbances. Many of the regular pathological symptoms, e.g. circulatory failure, fatty changes in the heart, interference with its action and haemorrhage into the adrenals, are also capable of being traced back to a biochemical origin, whilst the last-named occurrence may obviously lead automatically to further chemical interference.

We have already shown [Peters & Cunningham, 1937] that the polyneuritic condition that occurs in the later stages is not due to induced vitamin B_1 deficiency, since the chemical changes associated therewith in the blood are not shown. Since there are no indications of lack of any other vitamins or dietary factors we conclude that the chemical disturbances are of a more profound origin. We have therefore turned our attention to a number of isolated tissue enzyme systems, and studied their behaviour in presence of diphtheria toxin.

The scope of our work embraces respiration, glycolysis, oxidations (including autoxidations) and phosphorus exchange.

Technique. For our work on respiration and oxidation we generally used the Warburg manometric technique, but macro- or semi-micro-analytical methods were substituted for the study of glycolysis and P interchange.

The toxin usually employed was a dried preparation containing 250,000 m.l.d. per g. (the gift of the Evans Biological Institute, to whom our thanks are due), free of preservatives, which had been found undesirable; it was used in large excess of the m.l.d., usually 1 or 2 mg.

Enzyme preparations were obtained as far as possible from heart muscle, which is known to be affected in diphtheria.

Experimental

Glycolysis

Finely chopped fowl's heart muscle was suspended in Ringer solution at *p*H 7·4 in a Krebs flask [Krebs & Henseleit, 1932], and incubated for 3–4 hr. at 38° in a stream of O_2 containing 5% CO_2. Flasks were set up in pairs, one containing toxin. Lactic acid was determined before and after incubation by the method of Friedemann *et al.* [1927].

Table 1 shows typical results from a large number of experiments. The increases in lactic acid per dg. of dried tissue are practically identical with and without toxin. Pyruvate was never detected in the mixture after incubation. Anaerobic experiments in an atmosphere of N_2 with 5% CO_2 also gave negative results.

Table 1. *Glycolysis*

2 g. tissue in 40 ml. Ringer medium (Ringer phosphate for Exp. 12). Glucose, 0·2%. Toxin 0·5 ml. (=25 m.l.d.) Burroughs Wellcome conc. solution, dil. 1 in 50.

Exp.	Incubation hr.		Tissue (g.) dry wt.	Lactic acid (mg.) Initial	Lactic acid (mg.) Final	Increase (mg./dg. dry tissue)
10	3	Control	0·3	1·7	2·4	0·29
		Toxin	0·3	2·0	2·1	0·03
12	3·5	Control	0·44	2·5	4·9	0·55
		Toxin	0·42	1·5	3·9	0·58

Tissue respiration

Slices of fowl's heart, suspended in phosphate Ringer at *p*H 7·4, were employed for a long series of manometric experiments in presence of glucose, glucose and citrate, glucose and lactoflavin, lactate, lactate and fumarate, and succinate. The manometer flasks were filled with pure O_2 and shaken for an hour at 38°. Parallel 'blank' experiments were run with the tissue but no substrate.

The results indicated that toxin caused no regular alteration in Q_{O_2}.

Oxidizing systems

A number of the oxidase systems that have been shown to exist in the body were set up *in vitro* and the effect of diphtheria toxin examined on each of them under various conditions depending on the particular enzyme being studied; these included succinic, lactic, malic, β-hydroxybutyric, α-glycerophosphoric and pyruvic dehydrogenases, and amine, amino-acid and adrenaline oxidases. The autoxidation of adrenaline in buffer was also studied.

Cozymase, when required, was prepared from yeast by a method described by Green (private communication), and further purified with CuCl [Euler *et al.* 1936]. We are indebted to Messrs Roche Products Ltd. for the gift of a sample of cocarboxylase for use in the pyruvic dehydrogenase experiments.

Acetone-dried preparations supplemented by diaphorase were used in a few experiments with lactate, malate and pyruvate.

Oxidations were generally carried out in phosphate buffer at *p*H 7·2–7·4 (except where otherwise stated) and an atmosphere of pure O_2. Toxin was added direct to the enzyme in some experiments, but in others it was mixed with the enzyme (and often cozymase as well) on the previous day and the mixture left overnight in the ice-box to attempt to allow for the 'latent period' observed with the toxin *in vivo*.

Tables 2–9 show typical results for all these series of experiments. The total number of experiments is indicated at the head of each table.

Lecithin. The effect of the toxin was studied on the oxidation of lecithin in presence of glutathione [Hopkins, 1925]. Experiments were carried out in acetate buffer at *p*H 4·6, which was found to be the optimum reaction. Preliminary contact with the toxin for varying periods was tried with each reactant. Other proteins, named in Table 10, were used for comparative experiments to ascertain if they produced any effects similar to that of toxin.

The final volume of liquid in the manometer flasks was always 2 ml., about 1 ml. of enzyme suspension being used and the other reagents added in the concentrations or dry weights given. If cyanide was present the precautions of Krebs [1935] were followed with respect to the CO_2-absorbing liquid in the inner cups.

Table 2. *Succinic dehydrogenase*

1 ml. enzyme [Stotz & Hastings, 1937] from heart of pig or sheep; 0·05 *M* phosphate, *p*H 7·4; 0·05 *M* succinate; 1 mg. toxin overnight with enzyme. Twenty-three experiments.

Exp.		O_2 uptake in 30 min. (μl.) Control	Toxin
15	No methylene blue	276	290
	Methylene blue, 0·008%	278	261
23	No selenite	339	342
	Selenite, $0·5 \times 10^{-3}$ *M*	19	17
16	HCN, $2·5 \times 10^{-3}$ *M*, methylene blue 0·063%	50	45

Table 3. *Lactic, malic and β-hydroxybutyric dehydrogenases*

1 ml. lactic [Green & Brosteaux, 1936], malic [Green, 1936, 2], or β-hydroxybutyric dehydrogenase [Green *et al.* 1937], from pig or sheep heart; 0·025% methylene blue, 0·015 *M* pyrophosphate; HCN, hydrazine or semicarbazide (0·015 *M*) as ketone fixative; toxin overnight with enzyme. Ninety-eight experiments.

Exp.	Substrate	O_2 uptake per hr. (μl.) Control	Toxin
46	Lactate	249	230
82*	Lactate	29	27
29	Malate	197	203
84*	Malate	24	24
79	β-Hydroxybutyrate	40	41

*Dried muscle enzyme with heart flavoprotein [Straub, 1939] as diaphorase.

O_2 uptake was not altered significantly by overnight contact between the toxin and the enzyme or enzyme-cozymase, presence or absence of pyrophosphate, or any particular ketone fixative.

Table 4. *α-Glycerophosphate dehydrogenase*

1 ml. enzyme [Green, 1936, 1] from guinea-pig skeletal muscle; 0·04 *M* α-glycerophosphate 2 mg. toxin; *p*H 7·2. Two experiments.

Exp.		O_2 uptake per hr. (μl.) Control	Toxin
1	No methylene blue	43	42
2	Methylene blue, 0·075%	170	168

Table 5. *Pyruvic dehydrogenase*

0·8 ml. enzyme [Lipmann, 1939] from skeletal or heart muscle; 0·2 *M* NaF, 0·04 *M* phosphate *p*H 7·2, 0·04 *M* pyruvate, 4 mg. adenylic acid (yeast), 3 mg. toxin; 1 mg. cozymase and 1 mg. cocarboxylase in side-bulb with pyruvate. Seven experiments.

Exp.		O_2 uptake per hr. (μl.) Control	Toxin
2		171	176
6	0·25 mg. lactoflavin (Roche) added	27	29

Table 6. *Amine oxidase*

1 ml. enzyme [Richter, 1937] from liver; 0·5 mg. toxin added in 0·9% NaCl, 0·001 *M* HCN as ketone fixative. Seven experiments.

Exp.	Substrate	O_2 uptake per hr. (μl.) Control	Toxin
4	Tyramine, 0·04 *M*	66	71
5	Adrenaline, 0·005 *M*	84	87

Table 7. *Amino-acid oxidase*

1 ml. enzyme [Krebs, 1933] from guinea-pig kidney; 0·1 *M* hydrazine, 1 mg. toxin added solid or in 0·9% NaCl, *p*H 7·4. Fourteen experiments.

Exp.	Substrate	O_2 uptake per hr. (μl.) Control	Toxin
4	*d*-Alanine, 0·1 *M*	95	82
11	*l*-Cysteine, 0·025 *M*	214*	222*
13	*l*-Cysteine, 0·025 *M* (autoxidation—no enzyme)	158	169

* Total O_2 uptake.

Table 8. *Adrenaline oxidase*

Enzyme [Blaschko *et al.* 1937] used crude or purified by precipitation with 30% alcohol or isoelectrically at *p*H 5·0; 0·013 *M* adrenaline, 0·5 mg. toxin, *p*H 7·4. Sixty-two experiments.

Exp.	Enzyme	Addition	O_2 uptake per hr. (μl.) Control	Toxin
35	Crude	2 mg. glutathione	302	303
28	Alcohol ppt.	HCN, 0·001 *M*	35	45
51	Ppt. at *p*H 5·0	0·16 mg. ascorbic acid	174	178

The presence of HCN, glutathione, or ascorbic acid, or of any two of these together, caused no significant alteration in the effect of toxin.

Table 9. *Autoxidation of adrenaline*

Adrenaline 0·006 *M* in 0·1 *M* phosphate (Na and K salts), *p*H 7·4; 1 mg. toxin added in water. Eighty-seven experiments.

Exp.		O_2 uptake per hr. (μl.) Control	Toxin
77		208	211
14	HCN, 0·001 *M*	96	104
42	Normal buffer	308	199
	Na buffer free of K	223	186
49	Normal buffer—toxin in isotonic NaCl	223	164
	K buffer free of Na—toxin in isotonic KCl	121	124

Table 10. *Oxidation of lecithin*

1·6 ml. lecithin suspension (ox liver) in water; toxin and other proteins added solid; glutathione (4 mg. except where otherwise stated) in water in side-bulb, *p*H 4·6. Forty-four experiments.

Exp.	Toxin (mg.)	O_2 uptake per hr. (μl.) Control	Toxin
30	2	105	140
(Diphtheria antitoxin, 131; toxin—antitoxin (overnight contact), 144 μl. per hr.)			
32	2	143	147
(Egg-white, 46; wheat-gluten, 110 μl. per hr.)			
36	2	76	100
(Antitoxin, 89; toxin—antitoxin (mixed direct), 88; cryst. ovalbumin, 88 μl. per hr.)			
33	2	116	177
	1	116	170
	0·5	116	156
	0·25	116	148
41	0·25 Glutathione, 1 mg.	67	79
	Glutathione, 0·5 mg.	73	106

In individual experiments in each group occurred considerable random variations from +50 to −50%, with and without toxin, but taken collectively the results with toxin showed no real deviation from the normal under most of the conditions studied.

The net O_2 uptake for every enzyme varied with each individual preparation, but duplicate experiments with a fresh preparation showed good agreements in both the control and toxin series; with an uptake of 100 μl. or more per hour and the manometer taps frequently re-greased there was rarely obtained a deviation greater than $\pm 10\%$.

With lecithin and glutathione the toxin most frequently caused an acceleration (see Table 10). The accelerations sometimes produced by the other proteins were rarely of the same order as that from toxin.

In the autoxidation experiments with adrenaline where retardations were obtained, the most interesting results were those in absence of either Na^+ or K^+, as shown in Table 9; the retardation in presence of Na^+ and the negative effect with K^+ were too consistent to be due to experimental error. In later experiments, however, with both ions present, consistent retardation was not found, although external conditions were precisely the same.

Phosphorus exchange

Nucleotidase activity. The toxin was tested for nucleotidase activity by incubation for periods of 3 hr. in Krebs flasks with a mixture of nucleotides prepared by hydrolysis of vegetable sodium nucleate with alkali in the cold. Free phosphoric acid was determined colorimetrically before and after incubation, and control vessels were run without toxin. When toluene was present as antiseptic the increase in free phosphoric acid was virtually zero in both vessels.

Phosphorylation. The effect of toxin on three phosphorylation systems was investigated.

For the coupled oxidation-phosphorylation system of Needham & Pillai [1937], the enzyme was prepared from rabbit or guinea-pig skeletal muscle, or fowl's liver brei; the hydrogen acceptor was pyruvate, whilst both glycerophosphates and hexosediphosphate were used as donators. The mixed nucleotides used in the nucleotidase experiments, adenylic acid, or aneurin, served as P acceptor. Experiments were again of the macro-type, the mixtures being incubated for an hour at 38°; inorganic P was determined before and after incubation by the method of Fiske & Subbarow [1925]. Aneurin was found to interfere with the colorimetric determinations by forming a precipitate with the molybdate reagent, and was first removed by adsorption on kaolin.

Total inorganic P disappearance was in general very small, and was in most cases unaltered by toxin even after overnight contact. But with the enzyme suspensions from the muscles of one particular rabbit we obtained a series of results where the toxin caused an increase in P disappearance if left overnight in contact with the enzyme. Table 11 shows typical results from this series. We are inclined to think that these positive results originated in some degree from the particular animal, the treatment of which, however, differed in no way from that of the others except that it was killed by chloroform instead of by a blow on the neck. These active enzyme suspensions were also found to give a precipitin reaction with the toxin.

For investigation of the pyruvate-adenylic acid system of Lipmann [1939] (see Table 5), and the glucose-kidney-extract system of Kalckar [1939], O_2 uptake was measured manometrically and inorganic P afterwards determined in the contents of the flasks. In neither case was the toxin found to affect P esterification.

Table 11. *Phosphorylation of nucleotides*

2 ml. enzyme suspension from muscle of chloroformed rabbit; 0·01 *M* glycerophosphate*, 0·02 *M* pyruvate, 0·01 *M* fluoride, 0·03 *M* phosphate, *p*H 7·4; 5 mg. toxin, overnight with enzyme, 0·5 mg. mixed nucleotides. Total volume, 4 ml. Incubation, 30 min. at 38°.

		Phosphorus (mg.)		
Exp.		Before incubation	After incubation	Esterified
4	Control	5·26	4·65	0·61
	Toxin	5·71	4·17	1·54
8	Control	4·35	4·35	0·0
	Toxin	5·00	4·56	0·44

* B.D.H.—largely β- with some α-isomeride.

Discussion

The results of this investigation up to the point reached in these experiments indicate that the enzyme systems studied are not directly affected *in vitro* by diphtheria toxin. This may be regarded as conclusive in the cases of succinic, lactic, malic and β-hydroxybutyric dehydrogenases, but for the other systems some further investigation to a degree depending on the system concerned might be advantageous.

On the other hand, the symptoms observed in diphtheria which suggested these investigations are still unexplained. The interference with the action of the heart is apparently not the result of inhibition of the muscle oxidases. So far as lactic dehydrogenase is concerned our results form a corollary to those of Corkill & Ochoa [1934], who showed that a toxaemic liver will not permit the resynthesis of glycogen from lactate. It follows therefore that all lactate formed in the body in diphtheria has to be oxidized in the muscles, so that any interference with the action of the lactic dehydrogenase would lead to a pronounced lactacidaemia.

We have so far made no attempt to study the effect of toxin on the oxidation of fats, and it must be remembered that the other symptoms of diphtheria that might be traced back to a biochemical origin—ketonuria and accumulation of fat in the heart—can quite conceivably be the result of interference with these processes.

The 'latent period' between the injection of toxin *in vivo* and the manifestation of its effect constitutes another factor which must be considered in connexion with the chemical effects observed. It is apparently the same length (about 48 hr.) under all conditions. It is known that the toxin on injection passes direct into the muscle tissue, so that one of two processes, or possibly a combination of both, may take place during the latent period. Either the toxin may pass into the interior of the cells and seriously disturb their internal equilibrium by its presence, or a further chemical change of the toxin is necessary before it becomes actually toxic, and occurs in the tissues during the latent period. On the face of it, there is a strong argument against the first possibility in that the appearance of the symptoms is very rapid at the end of the period, whereas if the toxin passed slowly into the tissue it would do its harmful work as it proceeded, so that the symptoms should appear gradually. As regards the second possibility, there is no real proof that the 'toxin' as produced from cultures of *C. diphtheriae* is the actual toxic substance, since it is grown on synthetic media *in vitro*, and has never been isolated from diphtheritic tissue. Further, Pappenheimer [1938] has shown that the toxic properties are destroyed by acetylation

with ketene, the number of amino-groups acetylated corresponding closely with the number of ϵ-amino-groups of lysine in the molecule. He was careful to point out that the toxic properties might equally possibly be due to the spatial arrangement of the ϵ-amino-groups as to their actual presence, but whichever is the correct postulation the free amino-groups form a basis for further chemical changes to the molecule in the body. If such a reaction were to occur in the body, it is quite conceivable that the 'super-toxin' formed might affect these enzymes in a way which the substance grown *in vitro* would be unable to do.

A serious disadvantage with all these investigations is that up to the present a 'chronic' diphtheritic condition has not been achieved in any animal for *in vivo* study.

SUMMARY

Experiments have been carried out *in vitro* to study the effect of diphtheria toxin on tissue respiration, glycolysis and a number of dehydrogenase and phosphorylation systems under varied conditions.

The dehydrogenase systems for succinic, lactic, malic and β-hydroxybutyric acids have been shown conclusively to be unaffected by toxin, even after preliminary contact between the enzyme and toxin, whilst the indications are that other systems studied are also unaffected. Oxidation of lecithin by glutathione is to some extent accelerated. The effect on autoxidations is inconclusive.

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23. THE SYSTEM DEHYDROASCORBIC ACID-GLUTATHIONE

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THE ability of tissue extracts from plants and animals, either to inhibit the oxidation of ascorbic acid (hereafter 'AA'), or to reduce added dehydroascorbic acid ('DHA'), is well known. In his earliest experiments on AA (hexuronic acid), Szent-Györgyi [1928] showed that the peroxidase system of turnip juice would oxidize the natural AA in the juice when H_2O_2 was added, but that ascorbic acid was regenerated on standing. He also found that minced rat kidneys quickly reduced added DHA made by oxidizing AA with I_2, and that this reduction was performed in buffer alone by SH glutathione ('GSH'). Similar results have been obtained by Strachitskii & Rubin [1936], who found that DHA injected into living cabbage leaves was reduced and greatly increased the respiration, and by Goldstein & Volkensohn [1938], who showed that in tumour, placenta, spleen and embryo tissue, the initial oxidation induced by Cu or Fe is followed by reduction back to AA. Also, Golyanitskii & Belonosov [1936] demonstrated that DHA from the oxidation of AA by heat and air is reduced by inclusion in an alcoholic fermentation.

Following Szent-Györgyi's suggestion of the role of GSH, Mawson [1935] explained the protective action of tissue extracts in preventing the destruction of AA partly, at least, by their content of GSH, whilst de Caro & Giani [1934] and Barron *et al.* [1936] showed that GSH was capable of completely protecting AA from oxidation by tissues or by Cu.

Bersin *et al.* [1935], however, were unable to reduce DHA with GSH. Their results are unexplainable and do not tally with those of any other workers. In fact, some of the characteristics of the reaction have been investigated by several workers, notably Borsook *et al.* [1937].

Pfankuch [1934] was the first to describe catalytic reduction of DHA by SH compounds. He showed the presence, in potato juice, of a heat-labile substance, precipitable by protein reagents, which catalysed the formation of AA from DHA and cysteine. Similar findings were reported by Hopkins & Morgan [1936]. GSH was found to prevent the oxidation of AA by the ascorbic oxidase of cauliflower and cabbage juice, but was itself vicariously oxidized in the process. They proved that this was due to a factor in the juice which catalysed the reaction between DHA and GSH, the rate of the uncatalysed reaction being not nearly high enough to account for the protection. Copper was not able to catalyse the reaction, so that its presence in the juice would not account for the effect. Szent-Györgyi [1931] had noticed the vicarious oxidation of GSH in the presence of AA and cabbage juice, but had not recognized its enzymic character. Martin [1940] has recorded a similar vicarious oxidation of 'virus protein' in the presence of AA, which he attributes to the SH groups of the protein and a reaction of the Hopkins-Morgan type.

Reducing catalysts were also demonstrated by Lohman & Sanborn [1937] in the juice of peas and beans, and by Rubin *et al.* [1937] in *Hydrangea*, *Cyclamen*,

Zea etc. The former workers have also confirmed Hopkins & Morgan's report that Cu is not responsible.

Fujita & Numata [1938], using pumpkin juice, and Kertesz [1938], using the juice of cauliflowers and cucumbers, were unable to repeat the observations of Hopkins & Morgan, but these have since been confirmed and extended by Crook & Hopkins [1938], who showed the pronounced effect of *p*H and the difference in behaviour between cauliflower and cucumber. The latter contained very little, if any, of the reducing catalyst.

This paper is a preliminary report of the separation of the reducing factor in cauliflower juice from the associated oxidase and the 'resynthesis' of the protection system of Hopkins & Morgan. The work is not nearly complete, but it was felt that what has been done should be put on record as it is not being continued for the time being.

Experimental

Enzyme. The source of enzyme has been the florets of cauliflower and broccoli, both English and foreign. These were minced and pressed and the juice centrifuged, and sometimes fractionated before use. Care was also taken to prevent access of Cu and glass-distilled water used throughout.

Dehydroascorbic Acid. Great difficulty was experienced in evolving a satisfactory and rapid method for making DHA. Attempts to prepare it in the solid state by the method of Moll & Wieters [1936] were not satisfactory. The product was never sufficiently pure and the impurities inhibited the reaction. Unfortunately, there has been no opportunity to try out another method, the details of which were very kindly supplied by Prof. Hirst of Bristol University.

Oxidation of AA by the oxidase plus atmospheric O_2 was used in many cases, as were Cu plus atmospheric O_2, Cu plus H_2O_2, and I_2. The last was also unsatisfactory as the I^- ion inhibits the reducing system very strongly. The most satisfactory method finally evolved was to use a saturated aqueous solution of pure benzoquinone (recryst. from light petroleum B.P. $< 40°$) in amount just less than the equivalent of the AA so that no quinone remained in excess. Quinone was shown to be a strong inhibitor of the reducing reaction whereas the quinol produced had no such effect.

Reaction velocity. The reactions were followed by two sets of methods. In the earlier experiments, AA was estimated by titration; later by a photoelectric procedure. To follow the course of the oxidation, 10 ml. of solution containing 10 mg. AA, 2 ml. buffer (*M*/5 Sørensen or McIlvaine, *p*H 6) and 1 ml. enzyme solution were shaken on a machine to ensure thorough aeration. Samples were removed at stated intervals, the enzyme destroyed with trichloroacetic acid and AA estimated by titration with $N/100$ I_2. All measurements have been done at *p*H 6 as this value is near the peak of both oxidizing and reducing systems. Reduction of DHA was followed anaerobically as described by Hopkins & Morgan using 2·5 moles of GSH per mole AA (i.e. slight excess of GSH) and estimating AA by the micro-Tillman method [Birch *et al.* 1933; Hopkins & Slater 1935], because of the presence of GSH, which also reacts with I_2. Alternatively, measurements were made aerobically. If DHA had been prepared by shaking with enzyme, no further addition of juice was made. *M*/100 KCN was now used to inhibit the oxidase completely, and, as shown below, this has no effect on the reducing system. Later, all measurements of AA were made by the direct photoelectric method of Evelyn *et al.* [1938], modified for use with larger quantities of ascorbic acid. The AA solution was diluted to contain between 2 and 10 mg. AA per 100 ml. and a strong dye solution—one giving an initial reading of

about 18 on the galvanometer scale—was used. For these stronger dye solutions it was necessary to construct a series of calibration curves covering different initial readings, as the straight line relationship breaks down. They are, however, very rapidly and easily made. A Wratten No. 63 filter was found best.

When employing the photoelectric method, the use of trichloroacetic acid for stopping the enzyme action was discontinued, as it was found to interfere with the readings by altering the dye. Instead, the enzyme was destroyed by first making the solution 30 % with respect to acetic acid and later diluting to give the 5 % recommended by Evelyn *et al.*

Again, when DHA was made by oxidizing AA with quinone, the quinol formed rapidly reduced the dye, making titration methods useless. However, the rate of reduction is slow in comparison with that caused by AA. Evelyn *et al.* have shown that the latter is complete within 5 sec. The reduction of dye by AA alone is thus easily found by extrapolation of the galvanometer readings.

Nature of the dehydroascorbic acid

It was at first suspected that the DHA formed by enzymic oxidation of AA differed from that obtained by simple chemical oxidation. GSH, anaerobically and in the presence of the juice, always reduced the latter form at only 60–70 % of the speed at which it reacted with the former. When DHA is prepared by oxidation with a reagent, or by using Cu as catalyst, either the reduced reagent or Cu remains in the solution as an 'impurity' which could conceivably act as an inhibitor. Table 1 shows the average velocity of reduction, by GSH in the presence of the juice, of DHA made by various methods, together with the velocities resulting from the addition of the corresponding 'impurity' to enzymically oxidized AA. Except in the case of large amounts of Cu (which cause precipitation in the juice), these are all higher than the rates with 'chemical DHA'. Thus the greater part of the inhibition is not due to the presence of such substances.

Table 1. *Velocity of reduction of DHA by GSH*

DHA prepared by oxidation with	Reaction rate	Substance added to 'enzymic' DHA	Reaction rate
Enzyme	100	—	—
I_2—I^- ion still present	33	0·04 *N* I^-	80
I_2—I^- precipitated by Pb	72	0·02 *N* I^-	89
Cu (20 mg./100 ml.)—H_2O_2	40	—	—
Cu (20 mg./100 ml.)—H_2O_2, Cu removed by dithiocarbamate	61	Cu: 0·2 mg./100 ml.	100
Cu (1 mg./100 ml.)—O_2	66	1·0 mg./100 ml.	94
		2·0 mg./100 ml.	80
		20·0 mg./100 ml.	37
Quinone	66	Quinol: 10 mg./100 ml.	100

In an endeavour to obtain some evidence on the matter, an attempt was made to determine the specific rotation of DHA from each source. This proved very difficult and the results must be regarded with some suspicion, both because the rotation of DHA is very sensitive to changes in *p*H and concentration and nature of ions, and because periods of more than 20–30 min. enzymic oxidation cause appreciable opening of the lactone ring at the *p*H used. The short time means that unoxidized AA is present and must be allowed for in calculating the rotation: the juice, too, has a small blank.

The results are set out in Table 2. In Exp. 1, the AA control, the rotation was determined in the presence of NaOH equivalent to the AA, *M*/10 phosphate

Table 2. $[\alpha]_D^{20°}$ *of dehydroascorbic acid*

Exp. no.	Oxidizing agent	No. of repeats	Total specific* rotation	AA not oxidized (%)	Rotation of DHA*
1	Nil	3	23°	100	—
2	I_3	3	54°	0	54°±1°
3	I_2	4	39°	0	39°±1°
4	I_2	5	32°	0	32°±2°
5	$Cu\text{-}O_2$	5	26°	70	33°±2°
6	Oxidase	5	25·5°	70	31°±2°

* Calculated as AA.

buffer at *p*H 6, and 4% trichloroacetic acid, to represent the conditions which obtain in the Cu- and enzyme-oxidized solutions after addition of trichloroacetic acid. The figure found, 23° for the D line, compares favourably with that given by Herbert *et al.* [1933] for the Hg yellow line 578 mμ, with pure AA in weakly acid solution. Exp. 2 is included for comparison with the following values, and is pure AA oxidized with I_2 with no additions, i.e. the solution then contains only DHA and HI. The figure again is in good agreement with that of Herbert *et al.* In Exps. 4–6, AA was neutralized with NaOH and the oxidation carried out in phosphate buffer at *p*H 6, during 20 min.; HI, if present, was neutralized by adding the theoretical amount of NaOH; after acidification with 40% trichloroacetic acid to make the final concentration 4% the protein coagulum was centrifuged out if present, and the rotation determined. In the experiments with oxidase, a blank was run with the juice and all conditions the same but no AA present. The values shown in the table are corrected for this blank, which amounted to *c.* 20% of the total rotation. Exp. 3 was carried out as for Exps. 4–6, but the AA was not previously neutralized. It is included to show the effect of the extra amount of Na salts. After oxidation, an aliquot was taken on which unoxidized AA was determined by titration with I_2. The figures for the rotation of DHA in the presence of AA were calculated assuming that no opening of the lactone ring had occurred during the time occupied in oxidation. This is approximately the case, since Borsook *et al.* [1937] have shown that at *p*H 6 and 37°, only 15% of DHA is hydrolysed in 1 hr. Here the temperature was *c.* 18°, so that the inaccuracy thus introduced would be within the experimental error. For convenience, DHA is calculated as the equivalent amount of AA.

As will be seen, the rotation of the enzymically prepared form does not differ significantly from that of two chemically prepared modifications. We must conclude, therefore, that there is probably no great chemical difference between them.

The possibility of there being some type of loose complex necessary for the activation of DHA, and slowly formed in the juice, was considered, but allowing chemically prepared DHA to stand anaerobically with enzyme for some time before adding GSH was found further to decrease and not to increase the velocity.

The explanation appears to be that an inhibitor is present, and that this is precipitated when AA and the juice are shaken in air to prepare DHA (much protein is precipitated under these conditions). This, of course, does not occur when chemically prepared DHA is used. Weight is added to this conclusion by the finding that heating the juice to 38° for 20–30 min. precipitates a considerable amount of protein and at the same time increases its ability to catalyse the reaction by as much as 20%.

Separation of the reducing catalyst

It was found to be comparatively easy to obtain the reducing factor virtually free from the associated oxidase, and thus to show that they are distinct entities. Heating to 38–39° for 20–30 min. does not affect the oxidase and actually improves the reducing effect, as has been mentioned. This treatment removes much protein by denaturation and the muddy solution now becomes a clear yellow. On half saturating with ammonium sulphate, approximately 80 % of the oxidase is precipitated and only 30 % of the reducing factor. By raising the concentration of ammonium sulphate to 80 % saturation, the remainder of the 'oxidase' activity is thrown down, together with most of the reducing factor; the total recoveries of both oxidase and reductase being of the order of 90 %. Rapid dialysis of the 50–80 % saturated fraction causes loss of most of the remaining 'oxidase' activity (i.e. it is probably due to Cu^{++} ion), without appreciably lowering the activity of the reductase. Prolonged dialysis, however, affects the latter very considerably, the loss of activity being restored neither by heated juice nor by diffusate from raw juice. This indicates the inactivation of the reducing catalyst rather than the loss of a coenzyme-like compound. Repetition of the precipitation between the 50–80 % saturation limits removes more impurities and again gives a 90 % yield with no oxidase activity.

Properties of the reducing catalyst

The reducing system, either purified or in the plant, is rather labile. When plants are stored in the refrigerator for more than 2 or 3 days, they are generally found to contain little reducing activity. The purified preparation keeps its activity rather better, being reduced to about half in the course of a week. It is more thermolabile than ascorbic oxidase; exposure for half an hour to a temperature of 45° reduces its activity to half, whilst 55° destroys it entirely. On the other hand, 60° has almost no effect on the oxidase.

Extremes of *p*H reduce the activity. Even in buffer at *p*H 7·5 the activity is reduced to one third in 5 hr., and acids precipitate it completely [cf. Pfankuch, 1934].

Table 3. *Effect of inhibitors*

	Reaction velocity	
Final conc. of inhibitor	Reductase	Oxidase
Buffer alone at *p*H 6	100	100
$(NH_4)_2SO_4$: *M*/4	93	—
KI: *M*/25	78	100
M/50	89	100
Cu^{++}: 3×10^{-5} *M*	100	150
$1{\cdot}5 \times 10^{-4}$ *M*	94	—
3×10^{-4} *M*	80	—
3×10^{-3} *M**	37	—
Benzoquinone: *M*/1000†	61	—
Quinol: *M*/1000	100	—
M/200	100	—
Dithiocarbamate: *M*/1000	100	80
KCN: *M*/1000	100	0
M/100	100	—
H_2S: *M*/1000	100	0
M/10	110	—
NaF: *M*/1000	86	—
M/100	83	100
Na iodoacetate: *M*/1000	83	100
M/100	30	100

* Some precipitation of protein. † Added before GSH.

In neither case is the effect reversible. The *p*H-optimum of 6·7–6·8 given by Crook & Hopkins is confirmed; see Fig. 1. The activity becomes very low below *p*H 4 and almost impossible to measure above *p*H 8 because of the rapid hydrolysis of DHA.

The effects of various inhibitors are set out in Table 3, the results for ascorbic oxidase being included for comparison. As will be seen, the reducing catalyst is fairly sensitive to neutral salts; e.g. even moderate concentrations of ammonium sulphate inhibit somewhat, whereas the oxidase is not at all affected. Similarly, quite small concentrations of Cu^{++} ion inhibit a little, although the effect of the strongest concentration shown can be explained, to some extent at least, by the fact that this amount of Cu is sufficient to fix about 40 % of the GSH present, if co-ordinated GSH is assumed to be no longer available for reduction. Such typical inhibitors of the oxidase as KCN and H_2S, which act in $M/1000$ concentration, do not affect the reducing system even at ten times that concentration. In fact the latter speeds up the reaction, apparently being able to act in somewhat the same manner as GSH.

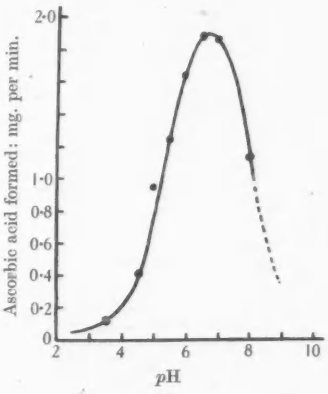


Fig. 1.

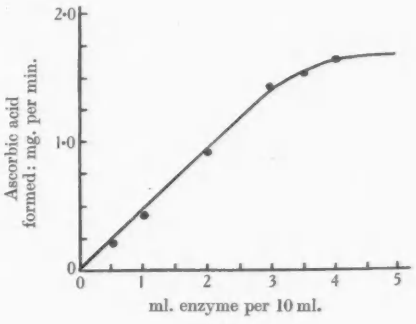


Fig. 2.

Fig. 1. *p*H-velocity curve for reductase. $M/5$ phosphate-citrate buffer, 1 mg. per ml. dehydroascorbic acid, 4·5 mg. per ml. glutathione (2·5 moles per mole of dehydroascorbic acid), 0·6 mg. per ml. quinol (quinone was used to make dehydroascorbic acid), purified enzyme preparation.

Kinetics

The initial velocity of reaction is proportional to the concentration of catalyst preparation over a wide range, the rate falling off at higher concentrations (Fig. 2—unpurified enzyme). It has not been possible to obtain adequate curves of velocity as a function of concentration of DHA and GSH owing to a shortage of the latter material, but assuming DHA to be the substrate rather than GSH (an assumption only valid for the purpose of this preliminary calculation), the Michaelis constant has been found in a few preliminary experiments to be of the order of $2 \times 10^{-3} M$ for the purified product. This is to be compared with a Michaelis constant for the oxidase of the order of $2 \times 10^{-6} M$.

The straight line kinetics described by Hopkins & Morgan for the reducing mechanism are less evident when purer preparations are used and when the relative concentration of GSH is reduced from 4 moles GSH per mole DHA as

used by Hopkins & Morgan [1936] and Crook & Hopkins [1938] to the 2·5 moles per mole here employed. The course of the reaction is initially linear, but when the concentration of GSH is low the curves begin to tail off fairly early (Fig. 3, curve *A*). With 4 moles GSH, this tailing off is hardly perceptible at the concentrations of other reactants here used (Fig. 3, curve *B*). However, when the uncatalysed component of the reaction is allowed for, even 4 moles of GSH do not give a high enough concentration to prevent tailing off. The reaction is obviously extremely complicated, and as its measurement is made more difficult by the simultaneous occurrence of the uncatalysed reaction as well as the hydrolysis of DHA to 2–3-diketogulonic acid, the reasons for this behaviour have not yet been elucidated. Fortunately, the rate of the last-mentioned reaction is sufficiently slow at the *p*H used not to interfere unduly with the significance of the curves given.

Fig. 3, curve *C*, shows a typical curve for the course of the catalysed reaction, this being obtained from curve *A* by subtracting the values of curve *D*, which shows the course of the uncatalysed reaction under the same conditions.

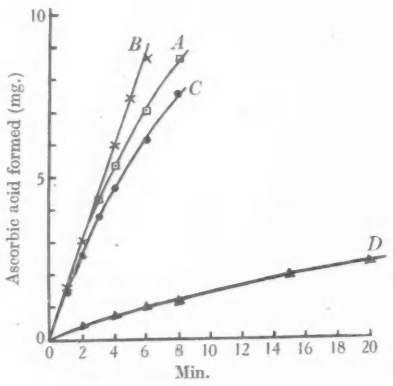


Fig. 3.

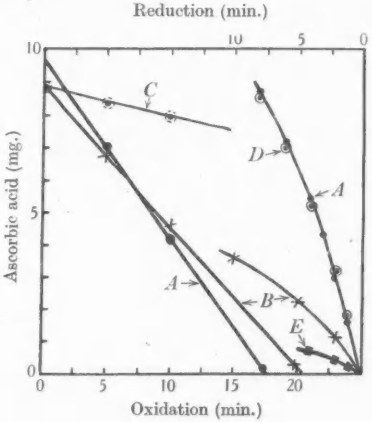


Fig. 4.

The association of this enzyme with ascorbic oxidase in the protective mechanism for ascorbic acid discovered by Hopkins & Morgan was illustrated in two ways. Firstly, by roughly separating the system into two parts by ammonium sulphate fractionation of the juice, testing each separately and then the recombined pair; secondly, by constructing a wholly 'artificial' protection system from oxidase and reducing catalyst.

In the first case, prepared juice was taken and the course of oxidation of AA and of reduction of the DHA thus formed was followed as by Hopkins & Morgan (Fig. 4, curves *A*). A portion of this juice was now separated into 0–50 and 50–80 % saturated fractions with ammonium sulphate. As noted above, the first contains the bulk of the oxidase and only about 20 % of the reducing factor. The course of the curves (*B*) thus obtained show a similar velocity for the oxidase, but a slow reducing reaction. The curve for the uncatalysed reaction (*E*) is included to show that, although the observed reduction of DHA is moderately fast, about half of it is accounted for by the simple chemical reaction. When the

50–80 % fraction is tested, its oxidase content is so low (Fig. 4, curve *C*) that the time required would be so long that much of the DHA would be hydrolysed. Hence this fraction was tested for the reductase in the following way. Oxidation

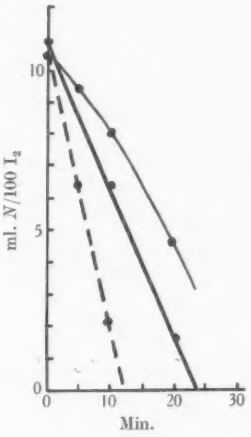


Fig. 5.

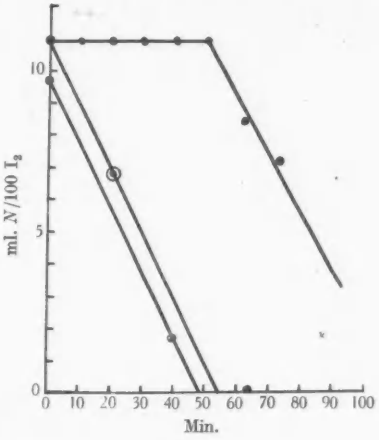


Fig 6.

Fig. 5. Upper curve: ascorbic acid in the presence of glutathione. Middle curve: glutathione. Lower curve: course of the oxidation of ascorbic acid alone.

Fig. 6. Upper curve: ascorbic acid in the presence of glutathione. Middle curve: glutathione. Lower curve: course of the oxidation of ascorbic acid alone.

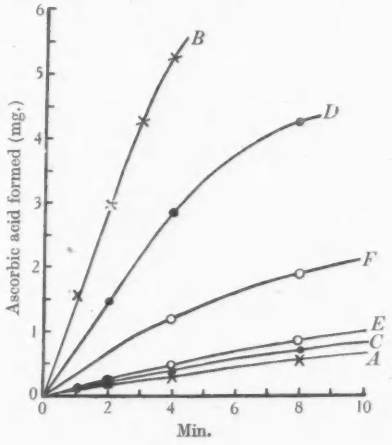


Fig. 7.

of AA was carried out by the oxidase fraction (curve *B*). Now, if the system is reconstitutable, addition of the equivalent amount of 50–80 % fraction should restore the full reducing power of the original juice, which it does (curve *D*, which is indistinguishable from *A*).

In the second set of experiments, cucumber juice was used as a source of the oxidase, as its content of reducing factor has been shown to be very low [Crook & Hopkins, 1938]. The oxidase was further purified by precipitation with 60% saturated ammonium sulphate, re-solution and dialysis. The reductase was purified as above. Concentrations of the reactants are expressed as their equivalents of $N/100$ I_2 solution, and the experiments were carried out at *p*H 6 as by Hopkins & Morgan. Fig. 5 shows the effect of having much oxidase and relatively little of the reducing factor. The AA is somewhat protected, particularly at the beginning, and the GSH is not oxidized as fast as is ascorbic acid alone. Note too that, as is to be expected, the sum of AA and GSH disappearing in a given time is approximately equal to the amount of AA which disappears in the same time in the absence of GSH. In Fig. 6 the relative concentrations are reversed, there being now relatively more of the reducing system. The result is a typical protection curve, the concentration of AA remaining unchanged until all the GSH has been oxidized at a rate which is the same as that for AA alone.

The behaviour of the reducing factor with cysteine and thiolacetic acid was tested. The courses of the reactions are shown in Fig. 7, *A* and *B* being the chemical and catalysed reactions with GSH, *C* and *D* with cysteine, and *E* and *F* with thiolacetic acid. GSH is obviously far more efficacious than either of the others, whilst thiolacetic acid gives a 'catalysed' reaction which is not much larger than the chemical one.

Discussion

That ascorbic oxidase and the reducing mechanism are separate entities seems to be beyond doubt. The fact that the reducing catalyst can be obtained in good yield virtually free from oxidase properties is the best evidence for this, but almost every other property is different. The Michaelis constant of the oxidase is of the order of a thousand times that of the reductase, the effect of inhibitors quite distinct and the thermolabilities different in the two cases. Finally, their relative activities in different samples of juice differ.

This being the case, it seems worth while to point out that Stewart & Stewart [1936] misquote Hopkins & Morgan when they ascribe the catalytic reduction of DHA to ascorbic *oxidase*, rather than to cauliflower press-juice. Similarly it is not difficult to see why Fujita & Numata [1938] were unable to repeat Hopkins & Morgan's results. They were using an oxidase preparation made according to Ebihara [1939], which would not be expected to contain much reducing catalyst; although they remark, 'if one adds too much oxidase, GSH is also oxidized'! It is surprising that they did not observe oxidation of GSH by DHA even chemically under the conditions used (*p*H 6 and 30°). Tauber [1938] too is wrong in stating that AA is not oxidized in the presence of GSH. The main point is that it can be reduced again faster than it is oxidized; but the O_2 uptake in the system is due to the oxidation of AA and not to the preferential oxidation of GSH.

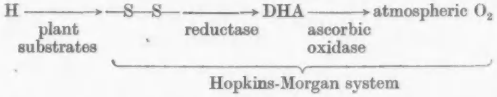
In this connexion, it is interesting to note that Snow & Zilva [1938] report that dialysed cucumber juice, containing less than 0·1 mg. AA per 100 ml., is capable of oxidizing GSH and cysteine directly, whereas dialysed cauliflower juice is unable to do so unless a trace of ascorbic acid is introduced [Crook & Hopkins, 1938].

It would be as well to consider the evidence as to the enzymic nature of the reducing catalyst, for the activity could well be ascribed to a simple chemical substance acting as a cyclic catalyst, particularly as the increase in reaction velocity is seldom greater than a factor of 7 or 8 (although this, of course, might

be explained by its occurrence in very low concentrations). The evidence must be indirect, as no preparation pure enough for chemical investigation has been made. The chief evidence in favour of its being an enzyme is (i) non-diffusibility through a cellophane membrane, (ii) great thermolability, (iii) precipitation by acids and other 'protein reagents' [cf. Pfankuch, 1934], (iv) the occurrence of a *p*H-optimum. The last, rather than a general tendency of the curve, would hardly be expected for a simple substance.

The relation of the reducing mechanism here described to other protective mechanisms for AA which have been reported in plants is not clear. Barron *et al.* [1936] showed that in many plant juices AA remained unoxidized on exposure to air. At the same time they found that GSH protected AA against Cu-catalysed oxidation, a result also found by Hopkins & Morgan. The amount required was very small and equivalent to the Cu present, i.e. GSH acts here by fixing Cu as a non-catalytic complex. Damodaran & Nair [1936] isolated a tannin from the Indian gooseberry (*Phyllanthus emblica*) which would inhibit the oxidation of AA in the press-juice. They concluded that its action depended on suppression of heavy metal catalysis since the protection could be 'over-ridden' by massive amounts of Cu. Kardo-Syssayava & Nissenbaum [1938] report a thermolabile stabilizer for AA in the structural parts of the tomato, but give no indication of the mechanism concerned in its functioning. The same comment is true of the findings of Giri & Krishnamurthy [1940], who were able by treatment with acetone to separate the press-juice of certain plants into a fraction containing ascorbic oxidase and one containing a substance preventing the oxidation of AA, even in the presence of added Cu. Apparently, the effect of this inhibitor was to depress the O_2 uptake of the system generally, so that they are probably not dealing with the Hopkins-Morgan system. The claim of Giri & Krishnamurthy that, previous to their publication, there has been no indication in the literature of the coexistence of oxidizing and protective factors for AA in plants is hardly substantiated by the literature reviewed in this paper.

The possibility that the Hopkins-Morgan system or a modification thereof is one plant analogue of the cytochrome system in animals is probably worth considering. While not wishing to suggest that it is so, it may be worth while setting out certain observations in its favour. Several authors, notably Ganapathy [1938] and Lohman & Sanborn [1937], have shown that —S—S— is reduced in plant juices. The former has also shown that this effect is abolished by heating, i.e. depends on further enzyme systems. Further, Povolockaja [1937] found that the O_2 consumption of the AA ascorbic oxidase system fully accounts for that of the whole germinating seed in legumes and cereals. Thus the Hopkins-Morgan system could conceivably act as the last link in a chain:



The criticism by Barron [1939] of the validity of the work of Hopkins & Morgan and Crook & Hopkins on the grounds that both the ratio AA : GSH and the total concentrations used were unphysiological loses much of its weight in the light of the above considerations. In fact the concentrations of AA used by Hopkins and his collaborators are well within the ranges exhibited by plants and are much below that occurring in such plants as the black currant and Hungarian red pepper. Neither were their ratios unphysiological, since, according to the work of Pett [1936], GSH and AA occur in potatoes in a molar ratio of approximately 2 : 1.

More recent work, such as that of Ganapathy & Sastri [1938], who showed that papaya and similar fruits contain as much as 0·3 % of GSH, casts further doubt upon the validity of Barron's sweeping statement that 'there is very little glutathione in plants and a large amount of ascorbic acid'.

SUMMARY

1. The enzyme catalysing the reduction of dehydroascorbic acid by SH—glutathione has been separated from the associated ascorbic acid oxidase.

2. It is shown to be responsible for the protection of ascorbic acid by glutathione in cauliflower juice.

3. The fact that its activity varies in different plants has been mentioned.

4. Its properties and some preliminary observations on the kinetics of the reaction are described.

5. Its possible role as an 'end system' in plants is discussed.

6. Some indication is given that oxidation of ascorbic acid by the oxidase or by simple chemical reagents gives rise to the same form of dehydroascorbic acid.

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24. A NEW METHOD FOR THE DETERMINATION OF PHYTOHORMONE ACTIVITY

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In view of the widespread occurrence of phytohormones amongst the bacteria, yeasts and fungi, it would not be surprising to find that growth promotion in these micro-organisms is governed by a mechanism fundamentally similar to that obtaining in the higher plants. No evidence has been obtained [Boysen Jensen, 1932; Nielsen & Hartelius, 1932; Bonner, 1932; Bünning, 1934], however, that the phytohormones produced by micro-organisms play any role in their growth. Stimulation of a metabolic activity has, on the other hand, been recorded by Grace [1937], who found that in the fermentation of sugar solutions by baker's yeast, gas production (measured in a saccharimeter after 1 hour) was greatly accelerated as compared with the control by 1 part per million of α-naphthylacetic acid, and to a lesser extent by 5 p.p.m. In other experiments, $\frac{1}{10}$ p.p.m. gave considerable stimulation, and as little as $\frac{1}{250}$ p.p.m. had some effect. Concentrations higher than 5 p.p.m. had a repressing action. Similar responses were obtained with β-indolylacetic acid, its propionic and butyric homologues, and with the K salt of α-naphthylacetic acid, all of which substances are known to possess a stimulating effect on plant growth.

The adoption of some standardized procedure based upon the growth of yeast, or upon the stimulation of its fermentative power, would provide a new and eminently satisfactory method for the rapid testing of materials suspected of phytohormone activity. The work described in this paper was undertaken in order to ascertain the possibility of devising a sorting test along these lines.

Experimental

The yeast used throughout the work was an English top fermentation type ('D.C.L.' brand), and a separate sample was obtained fresh from the manufacturers for each experiment.

1. *Stimulation of fermentative activity*

A. *Grace's technique.* The exact conditions under which Grace carried out his experiments are not described in detail in his paper, but an attempt has been made to repeat the work under conditions as nearly identical as the description permitted. The saccharimeter technique possessed obvious inherent defects, and seemed fundamentally unsound especially in that no control could be exercised over the volumes of gas lost through the open limb. As a consequence of uneven and varying sedimentation of the yeast cells, the volumes of gas issuing up either limb showed wide variations in control experiments, the results of which were never sufficiently reproducible to warrant the use of the method for the testing of hormone solutions.

B. *Eudiometer technique.* This procedure eliminates the most serious defects of the saccharimeter method, and on theoretical grounds information of a more accurate nature should be obtained by its use. A wide range of experiments has been carried out in order to determine the conditions under which the stimulating effect was most pronounced.

(1) The following were placed in a round-bottom 50 ml. flask:

10 ml. water,[1]
10 ml. 10 % sucrose solution,
4 ml. yeast suspension (5 g. yeast/20 ml. water),
1 ml. hormone solution (replaced by water in control experiments),

the flask being then closed with a rubber bung carrying a delivery tube. Five flasks were prepared in this way, giving the following hormone concentrations: 0 (control), $\frac{1}{10}$ p.p.m., 1 p.p.m., 5 p.p.m., 10 p.p.m. The flasks were placed in a water bath at a constant temperature of 24°, and the CO_2 produced during the fermentation was collected in eudiometer tubes over saturated brine, the volumes being noted at intervals. After 24 hr. the reaction was very slow; each flask was shaken until no further bubbles of gas were evolved. Several series of control experiments (in which the 1 ml. hormone solution was replaced by 1 ml. water) were carried out in this way, whilst both β-indolylacetic acid and α-naphthylacetic acid were examined using the same yeast sample. Representative figures are quoted in Table 1.

Table 1

Figures are ml. CO_2 evolved.

Concentrations (p.p.m.) ...	Control experiments					β-Indolylacetic acid					α-Naphthylacetic acid				
	0	0	0	0	0	0	$\frac{1}{10}$	1	5	10	0	$\frac{1}{10}$	1	5	10
Time (hr.)															
1	14·5	10·5	15	13·5	14	11·5	15·5	12·5	11·5	10·5	7	8·5	10·5	8	8
2	45	35·5	47	40	47	35	42	36	33	29·5	28·5	30	35	27	23
3	74	60	75	72	78	60	67	62	58	56	58	53	59·5	56	49
4	98	77	93	98	104	78	86	80	76	73	77	78	83	74	70
5	112	94	112	117	124	92	100	97	90	90	93	99	105	94	81
6	123	107	126	131	136	103	113	108	102	100	—	—	—	—	—
24	173	169	168	169	170	166	177	177	178	173	178	176	179	180	177
24 (after shaking)	182	180	179	180	182	183	185	188	184	182	184	186	189	185	180

From these experiments it would appear that some stimulation does occur as a result of the presence of hormone, the optimum effect being with a concentration of 1 p.p.m., and that with concentrations of more than 5 p.p.m. there is a repressing effect. Neither stimulation nor repression, however, is very marked.

(2) The bios complex, normally responsible for growth promotion in yeasts, is to some extent synthesized by the organism itself. Thus there exists within the cells a quantity of growth stimulant varying in amount in different samples. In an endeavour to obtain the yeast free from bios and related substances, the yeast samples were washed with 0·9 % NaCl prior to use. The washing was repeated three times in each case, and the experiment then carried out as described under (1) above. Under these conditions neither stimulation nor

[1] An aqueous mineral salt solution of the following composition: $(NH_4)_2SO_4$ 0·2 %, KH_2PO_4 0·2 %, $MgSO_4, 7H_2O$ 0·05 %, $FeSO_4, 7H_2O$ 0·001 %, has been substituted in several experiments, but without any appreciable difference in the results.

repression was exhibited. Although the matter was not further investigated, this negative result with washed yeast tends to suggest that the promotion of fermentation may be dependent upon the presence of a system of substances whose activities are in some way closely inter-related. A similar conclusion has been reached by Kögl [1935] on the functions of the separate fractions of the bios complex in relation to yeast growth.

(3) The precise influence of O_2 on the reaction was investigated by carrying out the fermentation of a series of controls under both aerobic and anaerobic conditions.

In the aerobic experiments, individual charges, each in a 60 ml. flask, were composed of:

20 ml. water,
20 ml. 10 % sucrose solution,
8 ml. yeast suspension,
2 ml. water (replacing hormone solution in controls).

20 g. yeast were suspended in water to a total volume of 80 ml. This was aerated with a stream of sterile air during $2\frac{1}{2}$ hr., when 8 ml. of the suspension were used for each experiment. Figures for a representative batch of five flasks are given in Table 2.

Table 2. *Control experiments*

Figures are ml. CO_2 evolved

Time (hr.)	Aerobic technique					Anaerobic technique				
1	8	9	8·5	9	8	12	11	12	10·5	11
2	52	51·5	53	48	51	47	48	48·5	46·5	47
3	88	91·5	89·5	90	88·5	96·5	98	98	97	98·5
4	118	123	131	117	133	147	148	148	147	149

For the anaerobic series, charges of similar composition were prepared, but instead of aerating the yeast suspension it was treated with a stream of sterile N_2 during $2\frac{1}{2}$ hr., after which 8 ml. were transferred under strictly anaerobic conditions to each of the rest of the charges. For the purpose of this transfer, the apparatus shown in Fig. 1 was constructed. 20 g. yeast were thoroughly ground up with boiled water to give a total volume of 80 ml. suspension, which was introduced into flask *A*. A stream of N_2 from a cylinder, freed from O_2 by a series of alkaline pyrogallol washings, was bubbled through the whole apparatus during a period of $2\frac{1}{2}$ hr., clips *B* and *C* being open. Meanwhile, the ten 60 ml. flasks, each containing 20 ml. water and 20 ml. 10 % sucrose solution, had been heated to boiling for 2 min. to expel air, re-entry of the latter being prevented by Bunsen valves. The flask *A* was inverted, and the contents transferred to the graduated burette by N_2 pressure. The wash bottle *X* was reversed, the clips being closed during the process to prevent the entrance of air. Each flask in turn was attached to the burette (*Y* to *Z*), and 8 ml. yeast suspension introduced. The process to this point was strictly anaerobic, but the anaerobic introduction of hormone solution presents difficulty. The method adopted consisted in preparing the hormone solutions with freshly boiled water, and delivering the required 2 ml. by means of a pipette attached to an alkaline pyrogallol wash bottle. The flasks were then incubated in a water bath at 25° for 2 hr., the Bunsen valves being removed under brine, and the CO_2 evolved collected in eudiometer tubes. Typical results are shown in Table 2.

(4) The anaerobic technique was undoubtedly the most satisfactory of the various methods investigated, and was employed in the testing of the stimulating

power of a number of substances. The figures given in Table 3 are representative of those obtained in a wide range of experiments.

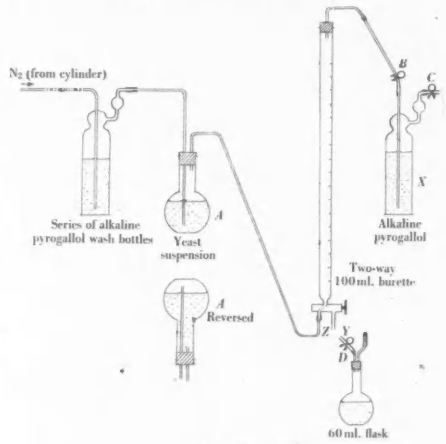

Fig. 1.

In the case of these four compounds both stimulating and repressing effects are obtained. With anthraquinone-1:5-disulphonic acid and anthraquinone-2-sulphonic acid, on the other hand, there is no appreciable effect in either direction.

Table 3

Figures are ml. CO_2 evolved

Concentration (p.p.m.) ...	β-Indolylacetic acid					α-Naphthylacetic acid				
Time (hr.)	0	$\frac{1}{10}$	1	5	10	0	$\frac{1}{10}$	1	5	10
1	14·5	15	15·5	13·5	14	12·5	13·5	17	14	12·5
2	40·5	42	41	43	43	41	43	45·5	41·5	41
3	85	87	92	88	87·5	82·5	87	92	85	85
4	123	132·5	137	138	129	126	130	141	140	139

Concentration (p.p.m.) ...	Phenylacetic acid					β-Naphthylacetic acid				
Time (hr.)	0	$\frac{1}{10}$	1	5	10	0	$\frac{1}{10}$	1	5	10
1	10·5	10	10·5	9·5	9·5	10	9·5	10·5	10	9
2	29	29·5	29·5	27·5	28	28·5	28	30	27	29
3	48	51	52	50	50·5	45	46·5	50·5	46	48·5
4	69·5	79	80·5	78	71·5	67·5	72	77·5	70	71
5	88·5	99	100·5	90	91·5	89·5	95	98·5	98·5	97
6	102·5	118	122	110	112	101	112·5	117	111	109
24	179	181	189	183	172	177	179	188	180	177·5

2. *Stimulation of growth*

In an endeavour to obtain optimum and, so far as possible, standardized experimental conditions, a wide range of influencing factors was examined:

I. Culture medium: (*a*) qualitative and quantitative composition, (*b*) character of the C source, (*c*) character of the N source, (*d*) *p*H, (*e*) influence of 'growth factors'.

II. External conditions: (*a*) temperature, (*b*) duration of experiment, (*c*) effect of O_2.

III. Inoculum: (*a*) biological nature of yeast, (*b*) physiological condition, (*c*) initial concentration.

The extensive work of Thorne [1933, 1; 1933, 2; 1936] in this field has been followed and, where necessary, supplemented along the lines required by the present problem.

A preliminary experiment was carried out in order to gain some idea of the accuracy of the direct cell-counting method of estimation of the yeast suspension. This is recorded in some detail since an identical experimental technique was adopted in later work with hormone solutions. 100 ml. of the culture medium:

KH_2PO_4	2 g.	Brought to *p*H 4·2 by addition of H_2SO_4
$MgSO_4, 7H_2O$	1 g.	
$CaSO_4$	0·2 g.	
$FeSO_4, 7H_2O$	0·1 g.	
$(NH_4)_2HPO_4$	2·5 g.	
Sucrose	25 g.	
Distilled water to	1000 ml.	

were placed in each of three 250 ml. conical flasks and sterilized by steaming during 1 hr. on each of three successive days. To each flask was added 1·0 ml. of a yeast suspension containing 1–2 g. yeast per 100 ml.; the seeding rate (i.e. initial count in the culture medium) was thus obtained as approximately 1–3 million cells per ml. The flasks were incubated at 25°, and counts were taken after three days. For the counts a Thoma-Zeiss haemocytometer was employed, all pronounced yeast 'buds' being arbitrarily counted as individual cells.

	Exp. 1	Exp. 2	Exp. 3
Initial count	117	89	97
	118	97	101
	113	99	99
	118	107	95
	126	91	98
	1,184,000	970,000	980,000
3 day count	633	399	589
	635	533	659
	604	648	431
	666	580	542
	6,360,000	5,400,000	5,500,000

For comparative purposes these figures may conveniently be expressed as 'multiplication rates', i.e. outcrop rate/seeding rate:

(1) 5·4, (2) 5·6, (3) 5·6.

The degree of accuracy shown in these preliminary controls was thus of a high order, and the investigation was extended to the use of known plant stimulants, and also to compounds hitherto not examined in this respect. In each case the following procedure was adopted.

Five 250 ml. conical flasks, each containing 95 ml. synthetic medium were sterilized by steaming. Hormone solutions of different concentrations (which had been prepared with freshly boiled water in order to obtain them as nearly sterile as possible without applying heat) were added in 4 ml. quantities, so that the final concentrations in the media were 0 (4 ml. sterile water added), $\frac{1}{10}$, 1, 5, 10 p.p.m. respectively. Finally, 1 ml. yeast suspension was added to each flask. After taking the initial counts, the cultures were incubated (water

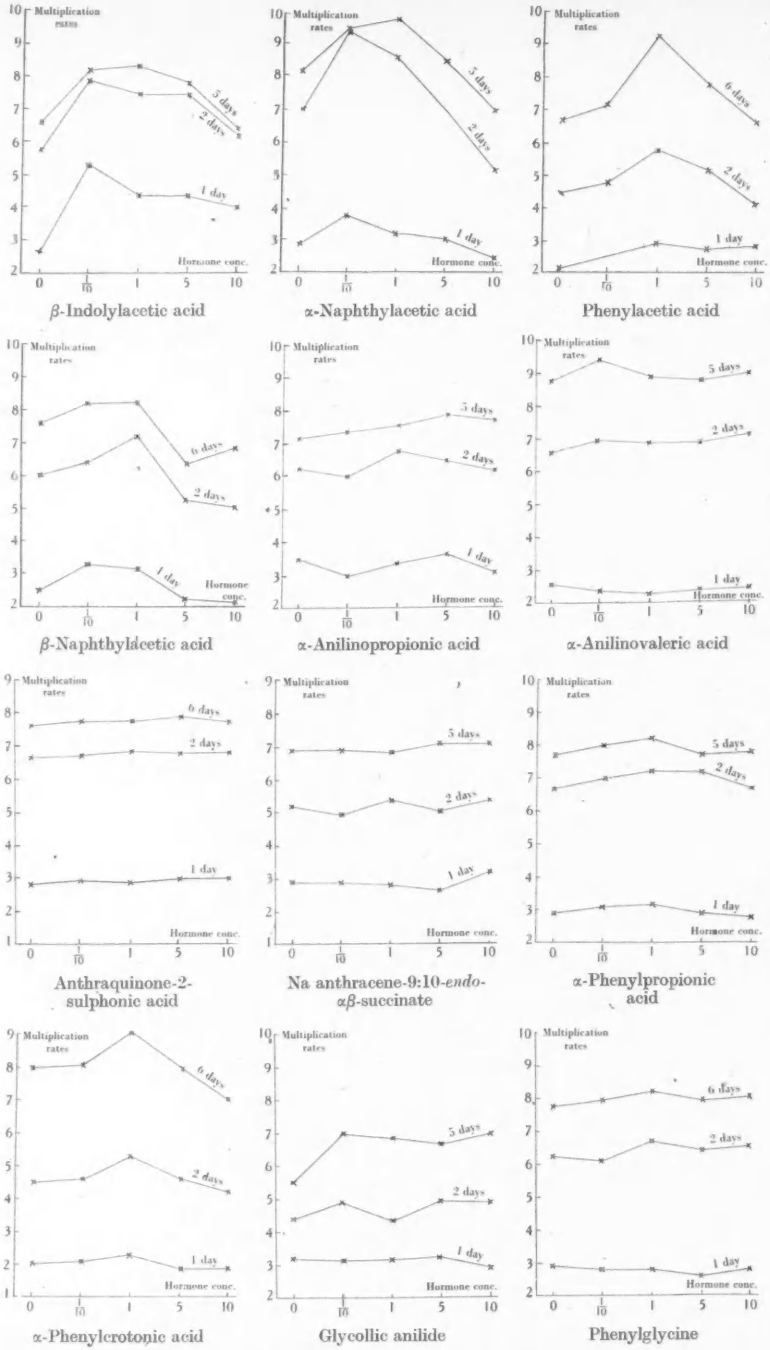

Fig. 2. Hormone concentration given in p.p.m.

bath) at 25°, and further counts performed after 1, 2 and 5 (or 6) days. Interpretation and comparison of the results obtained with the test substances are simplified by the graphical representations in Fig. 2.

In order to indicate the differences to be expected between inactive materials and those having pronounced or weakly stimulating effects, the results obtained with two such inactive substances (viz. anthraquinone-2-sulphonic acid, Na anthracene-9:10-*endo*-$\alpha\beta$-succinate) have been included.

Discussion

In an endeavour to obtain comparative data on the fermentation-stimulating activities of various substances, a wide range of experimental conditions has been investigated. The results have served to substantiate the main conclusions of Grace [1937], but the striking stimulating and repressing effects recorded by him have not been confirmed. Since it has been generally accepted that the auxins produced by yeasts and other micro-organisms have no effect on their growth, this stimulation of a metabolic activity of yeast is of considerable interest. The mechanism whereby the phytohormone action is exerted is not clear, but as preliminary washing of the yeast samples before use for fermentation nullifies the stimulating and repressing effect of these substances, it must be concluded that their action is linked with other factors which are removed or deranged by the washing process. Without further investigation of this point it would be premature to discuss the nature of the other factors involved. Went & Thimann [1937] have shown that a close inter-relation exists between β-indolylacetic acid, biotin and sucrose in their promotion of root formation on etiolated pea stems. Even the individual members of the bios complex are known [Kögl, 1935], as mentioned above, to be mutually interdependent for the optimum stimulation of yeast growth. This concept of a system of substances with interlocking functions is now established in many natural processes, and the possibility of a phytohormone-bios system cannot be overlooked in connexion with the stimulation of yeast fermentation.

Although fermentation has proved unsatisfactory as the basis of a sorting test for phytohormone activity, a standardized experimental procedure has been evolved, based upon the stimulation of yeast growth. The somewhat lengthy process of testing stimulating power against the higher plants is thus reduced to the few hours required for the reproduction of yeast cells.

Summary

1. Fermentation of yeast has been shown to be mildly stimulated by the presence of minute quantities of the known phytohormones, β-indolylacetic acid, α-naphthylacetic acid, β-naphthylacetic acid, phenylacetic acid.

2. It has not been found possible to standardize experimental conditions sufficiently to justify the use of the method as a sorting test for substances suspected of phytohormone activity.

3. The effect of washing yeast samples before fermentation is to nullify the stimulating effects ordinarily obtained with phytohormones. This is suggested as tentative evidence for the assumption of a phytohormone-bios system, the action of individual members of which is closely interdependent.

4. Contrary to accepted opinion, evidence has been obtained of the stimulation of yeast growth by phytohormones, and several compounds have been tested for phytohormone activity by observing their effects on yeast growth.

The author wishes to express his thanks to Prof. H. Raistrick in whose Laboratories this work has been carried out, and to Imperial Chemical Industries (Dyestuffs Group), Ltd., for their kind permission to publish the results given in this paper.

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25. THE CONVERSION OF LAEVULOSE AND FATTY ACID INTO GLUCOSE

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It has often been suggested that laevulose is converted into glucose in the animal body and a good deal of evidence has accumulated to support this view. Most of the evidence is indirect, but Cori & Shine [1936] claimed, by incubating liver slices with Ringer's solution, to have demonstrated the direct conversion of laevulose into glucose: they gave no experimental details. Goda [1937; 1938] showed that laevulose disappeared when incubated with liver or kidney slices suspended in Ringer's solution. In an attempt to prove more conclusively the direct conversion of laevulose into glucose, tissue slice experiments have been carried out and a complete carbohydrate balance sheet drawn up.

The possibility of glucose formation from laevulose was first shown by Reilly *et al.* [1898] who used phlorhizinized dogs. Mann & Magath [1922] found that laevulose was effective in hepatectomized dogs only if given some time before hypoglycaemic convulsions developed and suggested that glucose formation must be slow. Later, Bollman & Mann [1931] showed that such dogs could be kept alive for many hours without glucose if laevulose was given in sufficiently large amounts before the blood sugar was greatly lowered. It has been shown by Noble & Macleod [1924], Herring *et al.* [1924] and Corley [1929] that laevulose is not nearly so effective an antidote for preventing shock following the injection of insulin as glucose, unless it is given very soon after the insulin. Immediate protection would, it is presumed, have been afforded in all cases if laevulose could effectively replace glucose without being first converted into glucose or glycogen.

That the liver is the main site of the conversion is indicated by the work of Isaac [1920], Bodansky [1923], Lampson & Wing [1926], Corley [1929] and Mann & Magath [1922]. It has, however, been suggested that other tissues may have the ability to convert laevulose into glucose. Bollman & Mann [1931] showed that whilst laevulose was effective in preventing hypoglycaemic convulsions in hepatectomized dogs if given early enough, it was ineffective in eviscerated hepatectomized dogs, from which it was concluded that the intestines are a second site for the transformation. This is supported by Verzár & McDougal [1936] and Laszt [quoted by Verzár & McDougal, 1936] who consider that laevulose is converted into glucose in the intestine. These results have been contraverted by Steinberg [1927], Oppel [1929], Burget *et al.* [1932] and by Griffith & Waters [1936] who found that laevulose is utilized and can prolong life in dogs deprived of their liver and viscera. Goda [1938] showed that laevulose disappeared and glucose concentration was increased when laevulose was incubated with kidney slices. Steinberg [1927] showed that the isolated surviving skeletal muscle was able to utilize laevulose directly as efficiently as it could utilize glucose. This result supports McGuican [1908] and is confirmed by Griesbach [1929], Bornstein & Völker [1929] and Okamura [1938]. Griffith & Waters [1936]

also suggest that laevulose is utilized by the muscles and brain because it prolongs the life of eviscerated dogs. On the other hand, Maclean & Smedley [1912] and Stewart & Gaddie [1934] were unable to demonstrate any utilization of laevulose by the heart muscle of the dog, rabbit or frog, whilst a similar negative result was obtained by Ashford [1933] for brain tissue.

Experimental

The R.Q., O_2 uptake and lactic acid formation of freshly sliced tissue were determined by the method of Dickens & Šimer [1931], 100 mg. moist tissue slices (dried between filter papers) and 1·5 ml. of physiological salt solution [Krebs & Henseleit, 1932] to which laevulose was added, being used for each experiment. Total sugar and laevulose were determined before and after respiration by the method of Hagedorn & Jensen and a modification of that of Herbert [1938] respectively, whilst total carbohydrate was determined by the method of Ochoa [1930]. The respiration time in every case was 2 hr. The carbohydrate oxidized was calculated from the R.Q. and the O_2 uptake. Liver, kidney, brain, skeletal and cardiac muscle, spleen, and intestinal muscle and mucosa of the rat were the tissues examined. A typical balance sheet for liver slices is shown in Table 1.

Table 1. *Balance sheet for liver slices*

O_2 uptake	426 μl.	
R.Q.	0·78	
CO_2 equivalent of lactic acid formed	146 μl.	
	Before (mg.)	After (mg.)
Laevulose	2·33	1·26
Total sugar of medium (as glucose)	3·47	3·92
Total carbohydrate of tissue (as glucose)	1·25	0·45
Glucose converted into lactic acid	—	0·59
Carbohydrate oxidized (as glucose)	—	0·12
Total carbohydrate accounted for	4·72	5·08
Laevulose disappeared	1·05 mg.	

Table 2 shows the results obtained with the various tissue slices. In the first experiments, in which laevulose was the only sugar present in the medium, a big leakage of carbohydrate from the tissue into the medium occurred. This was common to all experiments in which the medium did not contain glucose and so in the subsequent experiments glucose as well as laevulose was present in the medium.

These experiments clearly show the direct conversion of laevulose into glucose by the liver and the kidney, since the amount of laevulose which disappeared is greater than the sum of the carbohydrate oxidized and that converted into lactic acid. All the remaining tissues examined failed to bring about the conversion, the amounts of laevulose which disappeared being negligible. This is especially interesting in the case of the intestine, since it contraverts the conclusions, from indirect evidence, of Bollman & Mann [1931], Verzár & McDougal [1936] and Laszt [quoted by Verzár & McDougal, 1936]. That the conversion is an aerobic reaction is shown by Exp. 14, in which liver slices were incubated with the usual laevulose-containing medium in an atmosphere of N_2 without the disappearance of any laevulose. It may be mentioned here that many attempts have been made, without success, to prepare an active liver brei which would bring about the conversion of laevulose into glucose (or glycogen). No stress can be laid on the R.Q. in the experiments with muscle and mucosa, since the

Table 2

Exp.	O_2 uptake μl.	R.Q.	μl. CO_2 equiv. of lactic acid formed		Laevulose mg.	Total sugar (medium) mg.	Total carbohydrate (tissue) mg.	Glucose converted into lactic acid mg.	Carbohydrate oxidized mg.	Total carbohydrate accounted for mg.	Laevulose disappeared mg.
						Liver					
6	357	0·87	85	B	2·53	4·35	0·63	—	—	4·98	—
				A	1·28	4·25	0·85	0·34	0·24	5·68	1·25
				(8 other exps. gave closely similar results)							
						Kidney					
13	740	0·81	68	B	2·08	3·42	0·68	—	—	4·10	—
				A	1·14	2·80	0·55	0·28	0·31	3·94	0·94
				(3 other exps. gave closely similar results)							
						Liver in an atmosphere of N_2					
14	−24	—	109	B	3·02	3·78	0·40	—	—	4·18	—
				A	2·92	3·78	0·45	0·44	Nil	4·67	0·10
				(1 other exp. gave a closely similar result)							
						Brain					
16	236	1·09	28	B	2·74	3·55	0·20	—	—	3·75	—
				A	2·69	3·20	Nil	0·11	0·29	3·60	0·05
				(2 other exps. gave closely similar results)							
						Skeletal muscle					
20	44	(1·56)	Nil	B	2·75	3·95	0·62	—	—	4·57	—
				A	2·75	4·10	0·67	Nil	0·05	4·82	Nil
				(1 other exp. gave a closely similar result)							
						Duodenum (muscle and mucosa)					
22	157	1·07	125	B	2·53	3·37	0·80	—	—	4·17	—
				A	2·42	3·05	0·50	0·50	0·19	4·26	0·11
				(1 other exp. gave a closely similar result)							
						Duodenum (mucosa)					
25	60	(0·42)	100	B	3·30	3·92	0·23	—	—	4·15	—
				A	3·25	4·08	0·27	0·40	Nil	4·75	0·05
				(2 other exps. gave closely similar results)							
						Cardiac muscle					
27	20	(1·55)	4	B	3·00	3·90	0·30	—	—	4·20	—
				A	2·86	3·80	0·25	0·01	0·02	4·08	0·14
				(1 other exp. gave a closely similar result)							
						Spleen					
29	366	0·80	89	B	3·14	3·47	0·25	—	—	3·72	—
				A	3·06	2·98	0·25	0·36	0·15	3·74	0·08
				(1 other exp. gave a closely similar result)							

B. Before respiration. A. After respiration.

Table 3

Exp.	Tissue		Laevulose mg.	Total sugar (medium) mg.	Total carbohydrate (tissue) mg.	Glucose converted into lactic acid mg.	Carbohydrate oxidized mg.	Total carbohydrate accounted for mg.	Laevulose disappeared mg.	Apparent gain in carbohydrate mg.
					Rat on normal diet					
30	Liver	B	2·39	4·22	1·38	—	—	5·60	—	—
		A	1·41	5·27	0·43	0·40	0·37	6·47	0·98	0·87
	Kidney	B	2·45	3·40	0·42	—	—	3·82	—	—
		A	1·38	2·80	0·42	Nil	0·87	4·09	1·07	0·27
					(2 other exps. gave closely similar results)					
					Rat on high carbohydrate diet					
33	Liver	B	3·03	4·27	1·90	—	—	6·17	—	—
		A	1·44	4·92	1·63	0·38	0·22	7·15	1·59	0·98
	Kidney	B	2·58	3·62	0·45	—	—	4·07	—	—
		A	1·44	2·70	0·45	0·61	0·28	4·04	1·14	−0·03
					(1 other exp. gave a closely similar result)					
					Starved rat					
35	Liver	B	2·31	3·50	0·45	—	—	3·95	—	—
		A	1·17	2·87	0·45	0·41	0·31	4·04	1·14	0·09
	Kidney	B	2·19	3·72	0·50	—	—	4·22	—	—
		A	1·03	3·50	0·40	0·15	0·12	4·17	1·16	−0·05
					(2 other exps. gave closely similar results)					

respiratory exchange was too small for accurate measurement. Had conversion of laevulose into glucose occurred at the same rate, proportional to the O_2 usage, as in liver, it would have been detectable by the direct estimations of laevulose, but nothing can be said as to the production of 'new' carbohydrate in these experiments.

It will be noticed that in all the experiments with liver slices, including those under anaerobic conditions, there was an apparent gain in carbohydrate, a phenomenon which was found with no other tissue except duodenal mucosa. Such an increase in total carbohydrate when liver slices were incubated with Ringer's solution had been previously reported by Gemmill & Holmes [1935], who because of the low R.Q. obtained with liver slices from butter-fed rats, attributed the increase to a conversion of fat into carbohydrate. This was criticized by Cori & Shine [1935] who pointed out that the increase could be attributed to the glycerol and phosphoric ester fraction of the fat. Bach & Holmes [1937] showed that the formation of carbohydrate under their experimental conditions was partially inhibited by insulin and that this fraction appeared to be derived from amino-acids. The precursor of the remaining carbohydrate formed they again suggested was fat.

That the increase in carbohydrate was not due to fortuitous errors of analysis is shown further by Exps. 30–35 (Table 3) which represent simultaneous experiments with liver and kidney slices. In every case liver slices gave an increase in carbohydrate, whilst kidney slices gave an almost perfect balance.

Exps. 30–35 show that the gain in carbohydrate was greatest with the livers from rats which had been on a high carbohydrate diet, and was practically non-existent with the livers from starved rats.

There are several possible precursors of carbohydrate in such a system as that used in liver slice experiments, e.g. protein and protein derivatives, the glycerol fractions of the fats and phospholipins, whilst the formation of reducing trioses from hexoses would produce an apparent gain in carbohydrate.

Since, in liver especially, some of the 'lactic acid' may actually be aceto-acetic and β-hydroxybutyric acids, the figure for glucose converted into lactic acid is probably somewhat high, and so consequently is that for total carbohydrate accounted for at the end of the experiment. Even if the whole of the 'lactic acid'

Table 4

Exp. 38. Estimation of urea before and after the incubation of 100 mg. moist liver slices with 1·5 ml. medium at 37°.

mg. urea N			Glucose equivalent of increase (G/N = 6·25)
Initial	Final	Increase	
0·008	0·012	0·004	0·025
0·004	0·004	Nil	Nil
0·012	0·012	Nil	Nil
0·012	0·028	0·016	0·100

Exp. 39. Estimation of non-protein N. Conditions as in Exp. 38.

mg. non-protein N			Glucose equivalent of increase (G/N = 6·25)
Initial	Final	Increase	
0·140	0·135	Nil	Nil
0·267	0·275	0·008	0·05
0·180	0·200	0·020	0·13

were derived from non-carbohydrate sources, there would still, however, be an apparent gain in carbohydrate.

Only minute increases in urea or non-protein nitrogen could be detected after incubation (Exps. 38 & 39, Table 4), and these could only account for a small fraction of the observed gain in carbohydrate even assuming an optimum conversion of protein into carbohydrate, i.e. a G/N ratio of 6·25.

If hexoses are broken down to reducing trioses, i.e. triosephosphates, or if phosphoric esters are the actual substances which appear as 'carbohydrate' and so account for the apparent increase, there might be some disturbance of the inorganic P/organic P ratio and there should be some increase in the organic acid-soluble phosphate. It would be peculiar if such changes in phosphate were confined to liver tissue when other tissues are also metabolizing carbohydrate. Experiments show very little disturbance of this ratio and very little, if any, increase in organic phosphate.

Table 5

Exp. 40. Acid-soluble P estimations before and after the incubation of 100 mg. moist liver slices with 1·5 ml. medium at 37°. Rat on high carbohydrate diet.

	Initial		Final	
	Inorganic P mg.	Organic P mg.	Inorganic P mg.	Organic P mg.
(*a*)	0·0965	0·0265	0·0937	0·0400
(*b*)	0·094	0·047	0·097	0·044

In one of these experiments there is no evidence of any increase at all in the reducing phosphoric acid esters; in the other, the increase found in the organic acid-soluble phosphate would, if it were all present as newly formed reducing substance (i.e. triosephosphate), account for 0·09 mg. 'glucose' in the calculated balance sheet. If it were triosephosphate formed from a non-reducing precursor (e.g. glycerol or glycerophosphoric acid) this is then the maximum amount of apparent carbohydrate which could be accounted for; if it were formed from a reducing or potentially reducing precursor (glucose or glycogen) only half this would be a gain in 'carbohydrate'. In either case the amount is much less than the apparent gains in carbohydrate actually found. It appears then that only a small part of the 'carbohydrate' which appears during the incubation of liver slices can be derived from protein and amino-acids or by an increase in reducing power due to formation of reducing phosphoric esters. It remains to consider the possibility of carbohydrate formation from fat.

In studying the changes in the fat during incubation, the fat was extracted from the tissue and medium by a mixture of alcohol and ether (3 : 1). Eventually the fatty acids were weighed after a treatment involving saponification, acidification with H_2SO_4 to liberate the fatty acids and their final extraction with light petroleum (B.P. 40–60°). The experiments reported in Table 6, show that in the case of liver slices, an increase in carbohydrate corresponds to a decrease in fatty acids whilst the kidney slices gave neither the apparent gain in carbohydrate nor any decrease in fatty acids. That the increase in carbohydrate cannot be attributed to the glycerol fraction of the fat is clearly shown by these experiments. Assuming the glycerol fraction of a fat to be approximately one-tenth of the molecular weight, then a loss 0·87 mg. of fat (as was found in Exp. 41) would correspond to only 0·09 mg. of carbohydrate derived from glycerol, whereas the actual gain was 0·52 mg.

Table 6

Exp.		Laevulose mg.	Total sugar (medium) mg.	Total carbohydrate (tissue) mg.	Glucose converted into lactic acid mg.	Carbohydrate oxidized mg.	Total carbohydrate accounted for mg.	Laevulose disappeared mg.	Increase in carbohydrate mg.	Total fatty acids mg.	Decrease in fatty acid mg.
				Liver. Medium containing laevulose and glucose							
41	B	3·25	4·12	0·28	—	—	4·40	—	—	5·68	—
	A	1·65	3·76	0·52	0·34	0·33	4·95	1·60	0·55	4·81	0·87
				(1 other exp. gave a closely similar result)							
				Liver. Medium containing glucose only							
45	B	—	4·60	1·22	—	—	5·82	—	—	4·34	—
	A	—	5·65	0·75	0·36	0·10	6·86	—	1·04	3·62	0·72
				(2 other exps. gave closely similar results)							
				Kidney. Medium containing glucose only							
47	B	—	3·00	0·35	—	—	3·35	—	—	3·31	—
	A	—	2·65	0·35	0·09	0·41	3·50	—	0·15	3·40	−0·09
				(1 other exp. gave a closely similar result)							
				Liver. Medium containing glucose only. Atmosphere of N_2							
49	B	—	3·40	0·77	—	—	4·17	—	—	2·75	—
	A	—	3·68	0·45	0·36	—	4·49	—	0·32	1·77	0·98
				(2 other exps. gave closely similar results)							

Discussion

Even if one adds together the amounts of carbohydrate possibly obtainable from protein and amino-acids, increase in phosphoric ester reducing substances, the glycerol of fats and the factor introduced by the formation of acetoacetic and β-hydroxybutyric acids making the total carbohydrate accounted for too high, there is still a very considerable amount of carbohydrate unaccounted for. The quantity involved is such that only the fatty acids would seem capable of being regarded as the source. Incidentally it may be remarked that the increase in carbohydrates with a corresponding decrease in fatty acids is not a property peculiar to experiments in which the medium contains laevulose, but is also found when the medium contains only glucose (Exp. 45).

It is evident that, of the factors considered, only the disappearing fatty acid is quantitatively sufficient to account for the apparent new formation of carbohydrate in liver slices. The further fact that in other tissue slices, in which no carbohydrate formation is detected, there is no loss of fatty acid, considerably strengthens the conclusion that fatty acids are converted into carbohydrate by liver.

There are, however, difficulties in accepting this conclusion without reserve. Thus the observed O_2 consumption is too low to allow the production of the observed carbohydrate from fatty acid together with the oxidation of the remainder of the fat which disappears. It is true that since the formation of carbohydrate and the loss of fatty acids seemed to occur in liver slices anaerobically (Exp. 49, Table 6) one may invoke an unknown hydrogen acceptor and imagine it to be functioning also in the presence of O_2. But this is not entirely satisfactory, and without further proof of the existence of such a hydrogen acceptor there remains a suspicion that some experimental error may have been overlooked.

It is important to remember that the carbohydrate balance sheets were calculated, at a time when the new formation of carbohydrate and the disappearance of fatty acid were unsuspected, on the assumption that the respiratory exchange represented complete oxidation. If fatty acid or anything else is converted into carbohydrate this assumption is unjustified and the calculations are wrong. They cannot be corrected, for, apart from the question of the anaerobic changes which may be occurring, recalculation would involve the assumption of the fatty acid conversion which the figures are designed to prove (or disprove). In any case, such a calculation leads to impossible results, which is not surprising in view of the apparent anaerobic changes in the carbohydrate and fat contents of the system. This error in the calculations does not affect the observation that carbohydrate (or at any rate, reducing substance) is formed in liver slices, for the increase in reducing power (calculated as glucose), plus the lactic acid formed is usually greater than the calculated carbohydrate loss by oxidation. In some experiments the actual reducing power of the system is definitely increased. Anaerobically, there is, of course, no loss of carbohydrate by oxidation, and the increase in reducing power is again real.

The apparent gain in carbohydrate has been observed by so many workers that it may be accepted, but since the gain is one of reducing power only, there is no complete proof that it is really due to formation of hexoses or polysaccharides. The exclusion of phosphoric ester production, does not amount to proof. Similarly the loss of fatty acid seems real and careful checking of the gravimetric analyses by a titrimetric procedure confirms the results and shows the disappearance of fatty acid to be due to complete removal of fatty acid molecules and not to a

shortening of the carbon chain. Incidently it is interesting to note that if the whole of the 'glucose converted into lactic acid' could be attributed to the keto-acids, it may account for the loss in fatty acids, since they are in the ratio of about 2·5 to 1, as are their molecular weights. The unexpected suggestion of an anaerobic conversion of fatty acid into carbohydrate with its corollary of a non-oxygen hydrogen acceptor is, however, disturbing, and indicates that much more work (which war conditions prevent) is required.

Summary

1. It is shown that the liver and kidney, but not the brain, spleen, cardiac muscle, skeletal muscle or duodenum, are capable of converting laevulose into glucose or glycogen. The conversion occurs only in the presence of oxygen and of intact liver cells.

2. All experiments with liver and duodenal mucosa showed an apparent gain in carbohydrate, whilst the other tissues did not.

3. The increase in carbohydrate was greatest with the tissues of rats which had received a high carbohydrate diet and least with those from starved rats.

4. The increase in carbohydrate corresponded to a decrease in fatty acid, but though the evidence is suggestive it is not regarded as proved that the fatty acid is converted into carbohydrate.

5. It is suggested that some hydrogen acceptor other than oxygen may, in these experiments, have been concerned in a disappearance of fatty acids and production of carbohydrate.

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26. OBSERVATIONS ON THE SERUM PHOSPHATASE OF CATTLE AND SHEEP

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The presence in blood plasma or serum of an enzyme capable of hydrolysing esters of phosphoric acid optimally at an alkaline reaction, has been known for many years [see McCollum & Hart, 1908], and it is now evident that this phosphatase is the phosphomonoesterase A_1 in the classification of Folley & Kay [1936, 1]. Thus, for example, Gutman & Gutman [1940] state that normal human blood serum contains very little 'acid' phosphatase. The amount of phosphatase present in blood is considerably less than in such tissues as intestinal mucosa, bone epiphysis, kidney and mammary gland [see Folley & Kay, 1936, 1]. Normal values of serum phosphatase in arbitrary units have been given for man and certain animals by Kay [1930, 1] and for man and dog by King & Armstrong [1934], but as yet only limited data have been presented for the activity of this enzyme in the blood of farm animals.

Serum phosphatase is raised above normal in certain generalized bone disorders in man, such as rickets (infantile, renal and adult), generalized osteitis fibrosa, osteitis deformans and obstructive jaundice [see Folley & Kay, 1936, 1], and it has also been claimed that it is raised in certain other diseases such as tuberculosis and carcinoma, but the evidence here is less conclusive. Since serum phosphatase values have proved of diagnostic value in disordered Ca and P metabolism in man it was hoped that the same might hold in farm animals, as was suggested, on the basis of a limited number of observations on sheep, by Auchinachie & Emslie [1933].

Before the possible diagnostic value of phosphatase determinations in farm animals could be assessed it was obviously essential to know the limits of probable variation in the normal animal. This paper records data obtained for normal cattle and sheep at the National Institute for Research in Dairying, Shinfield, and the Veterinary Laboratory, Weybridge, these institutions maintaining close co-operation throughout the work.

The data for cattle were in the first instance obtained at Shinfield during the years 1933–5 in the course of investigations into the possible relationship between blood and milk phosphatases [see Folley & Kay, 1936, 2] and were later supplemented by data obtained on other herds to see if certain peculiarities characteristic of the Shinfield cows were also shown under different conditions of herd management and by other breeds, and by some data from Weybridge.

During the period 1934–5 investigations which included serum phosphatase determinations were made at Weybridge into 'cripples' of lambs, a disease described by Lyle Stewart [1933] upon which extensive biochemical studies

were made by Piercy [1934, 1, 2]. It was hoped that serum phosphatase estimations would aid diagnosis of the bone disorder for which there is evidence in this disease, but the data showed wide variations uncorrelated with the incidence of 'cripples'. A large-scale experiment on the aetiology of 'pining' in Northumberland sheep which was jointly conducted by Mr W. Lyle Stewart and Dr A. P. Ponsford of Newcastle-upon-Tyne and Dr H. H. Green and Dr E. L. Taylor of Weybridge gave an opportunity of acquiring an additional large number of serum samples for phosphatase determinations. 'Pining' is a loose term for a wasting disease of sheep associated in Northumberland with helminth infection and mineral deficiency, possibly of cobalt. In all 240 ewes and lambs were bled, some of them several times, and over 900 samples were available for blood studies in various directions.

It should be stated here that these sheep were apparently normal and showed no evidence of 'pining' over the period in which the phosphatase data were accumulated. These data were supplemented by a further 220 samples from normal ewes in other flocks.

Experimental

All blood samples were obtained from the jugular vein, with the aid of partial stasis caused by a choke-rope in the case of cattle and by thumb pressure in the case of sheep.

Serum phosphatase was determined in both laboratories by the method of King & Armstrong [1934] which involves incubation for 30 min. at 37°. Two slight modifications were made: (*a*) sufficient phenol was added to the phenol reagent used for blank determinations to give a colour readily readable in the ordinary colorimeter, and (*b*) before making the colorimetric reading all tubes were immersed in the water bath at 37° for 45 min. to develop the blue colour.

Blood samples taken near either laboratory were left until the next day to ensure complete coagulation and the sera were then analysed. Samples taken at distant centres were sent by rail in special ice-boxes. When a batch of samples was too large to be dealt with in one day, the sera not analysed on the first day were kept for one or, on some occasions, two days in the refrigerator. It was previously found by one of us (W. M. A.) that storage of serum in the refrigerator at 2° for periods up to 4 days made no appreciable difference to its serum phosphatase activity. All haemolysed sera were discarded as unfit for phosphatase determination.

Results

The joint data are considered from a number of aspects, and for simplicity and economy of space the main features are presented in the form of distribution tables. In compiling these tables the values have been classified into groups covering 5 units for values from 0 to 30 units, 10 units for values between 30 and 50 units and thereafter in larger groups according to the data available. While all values were originally recorded to the first place of decimals, in the distribution tables they are, for simplicity, given to the nearest integer. Throughout this paper the term phosphatase refers to the phosphomonoesterase A_1 [Folley & Kay, 1936, 1] and all values are recorded in King & Armstrong units per 100 ml.

It will be noted that the number of samples exceeds the number of animals in the case of both cattle and sheep. This is due to the fact that 22 of the Dairy Shorthorn cows from one herd and 48 of the sheep from the 'pining' experiment at Newcastle-upon-Tyne were bled on several occasions. All the values so obtained have been included in the analysis of variability of results, since we

consider that this affords a better criterion of the extreme range of variation to be met with in normal animals. The alternative of entering the mean of the several values for each such animal in the distribution tables was considered and rejected, since this would tend to mask the extreme limits of variation. Only for investigation of the possible association between milk yield and serum phosphatase has the average value for each cow been taken.

Range of serum phosphatase in the apparently normal cow and ewe

For this study there were available 367 samples from 294 cows in 8 different herds maintained under varying systems of first-class dairy management, and 119 samples from the same number of Hereford cows kept under range conditions all the year round with the bare minimum of supplementary feeding in winter. For the ewe there are 555 samples from a number of pure-bred and cross-bred flocks. The overall ranges of values were 0·3–114·3 units for cows and 3·0–166·1 units for ewes. The summarized distribution data are given in Table 1.

Table 1. *Distribution of serum phosphatase values, actual and on percentage basis, in normal cows and ewes*

Serum phosphatase units	Dairy breeds		Herefords		All cows		All ewes	
	No.	%	No.	%	No.	%	No.	%
0·0– 4·9	59	16·1	38	31·9	97	20·4	11	2·0
5·0– 9·9	117	31·9	60	50·4	177	36·4	139	25·1
10·0–14·9	60	16·3	12	10·1	72	14·8	146	26·2
15·0–19·9	27	7·4	1	0·8	28	5·8	66	11·9
20·0–24·9	22	6·0	3	2·5	25	5·1	70	12·6
25·0–29·9	17	4·6	3	2·5	20	4·1	37	6·7
30·0–39·9	20	5·4	2	1·7	22	4·5	40	7·2
40·0–49·9	20	5·4	—	—	20	4·1	28	5·0
50·0–99·9	22	6·0	—	—	22	4·5	12	2·2
100·0 and over	3	0·8	—	—	3	0·6	6	1·1
Total no.	367		119		486		555	
Below 15 units		64·3		92·4		71·2		53·3
Below 40 units		87·7		100·0		90·7		91·7

The first thing to be noted in Table 1 is that, despite the wide overall range in each species, the greater proportion of values for both cows and ewes are under 40 units, though a small proportion of apparently normal animals show values above this limit, even exceeding 100 units in about 1 % of cases. Among the cows it will be seen that all values for the Hereford breed are below 40 units whereas there are 12·3 % of values over 40 units among the dairy breeds. Further discussion of this fact is left until later when possible breed differences will be considered.

Whereas in the data for cows 71·2 % of values are below 15 units and 19·5 % between 15 and 40 units, the corresponding figures for ewes are 53·3 and 38·4 % respectively. Considering now the values under 10 units, these are 56·8 % for cows and 27·1 % for ewes. These figures show that the scatter of values below 40 units is greater in ewes than in cows and suggests a species difference with the median value for ewes higher than that for cows.

The wide variations in apparently normal ewes and cows, particularly of the Dairy Shorthorn breed, revealed by the data in Table 1 make it evident that the diagnostic value of determinations of serum phosphatase in cases of suspected disorders of Ca and P metabolism in farm ruminants is likely to be extremely limited.

It is interesting to compare the normal ranges found by us in cows and ewes with the figures given by King & Armstrong [1934] for man and the dog. This is possible since the units were the same in both investigations. The normal range found by King & Armstrong for 24 normal human subjects between 19 and 42 years of age was 3·7–13·1 units, the majority falling between 5 and 10 units. For 21 dogs they found a wider range, two of them showing values of 27·4 and 32·1 units respectively, findings reminiscent of our results in ruminants.

Relation between serum phosphatase and age

Kay [1930, 1], Smith & Maizels [1932] and Stearns & Warweg [1933] reported that serum phosphatase was higher in children than in adults and similar age differences have been found in the rabbit and rat [Kay, 1930, 1] and dog [Bodansky, 1934]. The higher level in infancy may be associated with the more rapid skeletal development at this stage of life. Mulay & Hurwitz [1938] studied the plasma phosphatase in 300 human subjects ranging in age from the time of birth to 86 years and found comparatively high values between 1 and 15 years, a decrease between 15 and 25 years and thereafter a fairly constant level. Our data in general are in accord with the findings of these workers.

The data for Dairy Shorthorn cows were classified among the age groups, 2–4 years, 4–6 years and over 6 years. To these three groups were added a small group of heifers 9–24 months old, and three sets of results obtained on the same population of calves at three age periods. These data are given in Table 2.

Table 2. *Distribution of serum phosphatase in different age groups of Dairy Shorthorn cattle*

Serum phosphatase units	Calves 8 weeks	Calves 17 weeks	Calves 27 weeks	Heifers 9–24 months	Cows 2–4 years	Cows 4–6 years	Cows over 6 years	Total of calves	Total of heifers	Total of cows	Total of cows and heifers
0·0– 4·9	—	—	—	—	1	3	1	—	—	5	5
5·0– 9·9	—	—	1	—	15	12	6	1	—	33	33
10·0–14·9	2	3	14	8	8	3	3	19	8	14	22
15·0–19·9	8	14	8	4	2	3	3	30	4	8	12
20·0–24·9	6	8	1	2	1	4	2	15	2	7	9
25·0–29·9	8	2	—	3	—	—	2	10	3	2	5
30·0–39·9	3	3	—	—	—	1	1	6	—	2	2
40·0–49·9	4	—	—	1	2	—	1	4	1	3	4
50·0–99·9	1	—	—	—	1	—	6	1	—	7	7
100·0 and over	—	—	—	—	1	—	—	—	—	1	1
Total no.	32	30	24	18	31	26	25	86	18	82	100

Among the calves, data for which are recorded in the first three columns, there is at first a wide scatter of values from 10 to 50 units with a subsequent tendency towards a closer scatter and a downward trend of values. The individual records of 24 of the calves which were bled on all three occasions show that 22 of them yielded diminishing values at successive samplings, while the values for the other 2 were singularly steady throughout the period. The heifers form a small intermediate group and the three age groups of cows show a preponderance of even lower values up to 6 years old, after which there is again a number of higher values. The groups of animals studied here are too small to warrant much discussion, but the data do suggest that in cattle, after maturity, there is not likely to be any regular trend in the serum phosphatase level. It is admitted that the composite picture of the relation between age and serum phosphatase shown in Table 2 is far from complete, and the data do not permit

the drawing of hard and fast conclusions. The only way to obtain a satisfactory picture of the change of serum phosphatase with advancing age is to study a large number of animals from birth to old age, a long and difficult task in the case of cattle.

In addition to the data on cattle there are similar data for sheep and these are summarized in Tables 3, 4 and 5.

Table 3. *Serum phosphatase data in four Suffolk × Dorset Horn lambs from birth to about 10 weeks old*

	Age in days							
Lamb no.	1	7	14	21	27	36	51	67
911	264	90	57	45	72	73	68	32
912	181	135	47	65	89	86	72	30
913	226	64	64	40	43	22	18	13
914	229	134	127	84	54	41	31	16
Average	225	106	74	58	65	55	48	23

Table 4. *Average values of serum phosphatase in 10 Cheviot lambs from 25 to 185 days old*

Average age in days	25	43	51	58	71	85	112	161	185
Range of phosphatase	64–165	60–127	41–150	41–115	32–77	27–73	11–27	9–34	5–33
Average phosphatase	105	84	92	83	50	40	20	20	13

Table 5. *Percentage distribution of serum phosphatase in 7 age groups of sheep from Newcastle 'pining' experiment*

Serum phosphatase units	5 months old 139 samples	9 months old 43 samples	13 months old 69 samples	17 months old 89 samples	3 years old 92 samples	3¾ years old 87 samples	4 years old 93 samples
0·0– 4·9	—	—	—	—	1·0	—	2·1
5·0– 9·9	4·3	2·3	1·4	16·9	21·7	6·9	42·0
10·0– 14·9	8·6	23·2	23·2	24·7	23·9	41·4	26·9
15·0– 19·9	15·1	23·2	31·9	16·9	20·7	18·4	5·4
20·0– 24·9	18·7	16·3	8·7	15·7	6·5	15·0	8·6
25·0– 29·9	15·1	23·2	10·1	7·9	7·6	5·7	3·2
30·0– 39·9	21·6	7·0	18·9	11·2	7·6	5·7	6·4
40·0– 49·9	12·9	2·3	1·4	1·1	6·5	4·6	2·1
50·0– 99·9	1·4	2·3	2·9	3·4	3·3	1·1	2·1
100·0–199·9	2·2	—	1·4	—	1·0	1·1	1·1
200·0–349·9	—	—	—	1·1	—	—	—
Under 15 units	12·9	25·2	25·6	41·6	46·6	48·3	71·0
Under 40 units	83·4	95·4	94·3	94·4	89·2	93·2	94·7

Tables 3 and 4 show that at birth the lamb has a very high serum phosphatase which falls very rapidly in some cases and more slowly in others, the fall continuing up to about 6 months of age. There is a decided difference in actual level in the two groups at corresponding ages but the trend is much the same in each group. These groups are too small to enable us to say whether or not this difference is likely to be connected with the breed.

It will be noted (see Table 5) that while the percentage of values under 40 units is fairly constant after 9 months of age in the sheep, there is a more or less steady increase in the number of values under 15 units, which rises from 12·9 % at 5 months to 71·0 % at 4 years. This shows a progressive condensing

of the scatter of phosphatase values with advancing age, a finding not observed in the smaller and differing cattle populations studied. The general picture emerging from the data on sheep resembles in part the finding in cattle but is more clear cut, possibly because the life of the sheep is shorter than that of the cow and a larger proportion of the life-span has been studied. The sheep data can furthermore be considered as of more significance than the data on cows in that the same population of sheep was studied over the last three ranges of age, whereas the data in Table 2 were obtained on three different and smaller groups of cattle. The possible existence of a species difference in the relation between age and serum phosphatase, nevertheless, cannot be entirely ignored.

The serum phosphatase level of the individual

In view of the wide overall range of values found among cattle and sheep it was considered important to know also what variability occurred from time to time in the same individual. A certain number of cows, ewes and lambs have been bled on a number of occasions and the full data available for these animals are presented in Tables 6 and 7.

Table 6. *Serum phosphatase values of 11 dairy Shorthorn cows at successive samplings*

Name of cow	1934			1935						Range of values	Average
	1. xii.	5. xii.	10. xii.	14. i.	23. i.	11. ii.	11. iii.	8. v.	18. xi.		
Campion 2	23	—	24	21	—	15	17	24	35	15– 35	23
Carlet 3	—	—	58	—	—	57	56	53	114	53–114	68
Lily 3	—	11	11	10	—	11	17	11	12	10– 17	12
Flora 1	—	—	9	8	—	9	9	—	—	8– 9	9
Linda 5	—	—	61	—	—	47	58	68	—	47– 68	58
Patricia	8	—	11	—	—	10	10	13	—	8– 13	10
Peach 3	48	—	44	48	—	43	53	70	63	43– 70	53
Peach 5	—	17	17	—	29	28	27	21	44	17– 44	26
Pippin	—	—	22	26	23	18	27	27	—	18– 27	24
Red Rose	—	—	9	—	—	8	9	12	12	8– 12	10
Rosalie	—	—	42	44	—	40	49	35	67	35– 67	46

From the data in these tables it appears that the general level of serum phosphatase in the normal adult individual remains sensibly constant over periods of the order of at least 1 year in the case of cows and 18 months in the case of ewes and lambs. An animal showing a low serum phosphatase activity at the first sampling has usually continued to do so with moderate fluctuations around the mean level, whereas an animal showing a high initial value always showed a high value. It is further clear that those animals showing high values, e.g. Carlet 3, Linda 5, Peach 3 and Rosalie in Table 6 and Lambs 46, 71, 75, and 136 and Ewe 26 in Table 7, also showed considerably wider fluctuations in serum phosphatase than animals showing a generally low level. As far as we are able to tell, the level of serum phosphatase of the individual, whether high or low, does not seem to be associated with any clinical abnormality. The wide variation in general level of serum phosphatase among apparently normal cattle and sheep is, however, a matter of great interest, and the possibility that changes in serum phosphatase might be associated with physiological conditions such as pregnancy and lactation was examined.

Table 7. *Serum phosphatase values in 41 sheep at 7 different samplings showing individual variation*

No.	Nov. 1936	Dec. 1936	Jan. 1937	Feb. 1937	July 1937	Nov. 1937	Mar. 1938	Range	Average
					1936 lambs				
2	7	14	9	11	12	13	10	7– 14	11
6	13	19	13	15	19	18	8	8– 19	15
12	40	35	30	23	30	33	17	17– 40	30
15	20	36	25	33	31	30	26	20– 36	29
39	20	16	16	12	16	12	11	11– 20	15
40	10	15	12	13	18	10	11	10– 18	13
44	8	17	10	10	15	7	8	7– 17	11
46	102	129	205	156	52	131	89	52–205	124
67	27	22	8	9	23	15	8	8– 27	16
71	46	120	38	55	38	72	44	38–120	59
75	122	146	57	119	—	19	—	19–146	93
77	13	17	13	9	15	11	9	9– 17	12
99	47	38	20	25	29	28	23	23– 47	30
101	26	19	14	13	29	14	11	11– 29	18
102	27	21	13	17	12	15	9	9– 27	16
108	35	30	14	16	21	17	8	8– 35	20
131	39	26	21	23	26	25	15	15– 39	25
134	50	19	13	9	19	16	11	9– 50	19
136	142	—	—	—	172	316	—	142–316	—
139	36	48	24	35	14	30	37	14– 48	32
142	68	46	15	28	—	40	25	15– 68	36
					1936 ewes				
19	17	18	14	10	14	9	8	8– 18	13
22	7	10	8	8	9	9	9	7– 10	8
26	110	166	62	135	51	113	114	51–166	107
32	41	49	31	25	45	29	37	25– 49	34
49	9	11	7	8	11	6	6	6– 11	8
52	29	15	15	14	37	21	16	14– 37	21
60	20	14	9	7	10	9	8	7– 20	11
62	52	34	21	22	43	25	15	15– 52	30
84	11	12	5	9	10	10	6	5– 12	9
86	17	9	14	15	—	3	9	3– 17	11
91	26	27	13	16	19	16	9	9– 27	18
96	42	25	19	17	25	22	21	17– 42	24
113	17	22	7	7	10	14	8	7– 22	12
120	44	26	23	28	36	33	22	22– 44	30
122	16	17	8	13	14	11	7	7– 17	12
124	43	17	13	24	23	22	15	13– 43	23
149	9	12	6	8	11	11	7	6– 12	9
152	9	8	5	5	9	6	6	5– 9	7
154	15	18	5	7	17	12	11	5– 18	12
156	17	20	10	7	19	7	9	7– 20	13

Serum phosphatase and pregnancy in the dairy cow

Wilson & Hart [1932] and Auchinachie & Emslie [1933] claim that serum phosphatase is lower in pregnant than in non-pregnant cows, while the latter authors also found the same to hold for ewes. Vermehren [1939] stated that in women serum phosphatase is increased in the latter half of pregnancy, reaching a maximum just before parturition and falling sharply immediately *post partum*. He further states that the level during lactation is somewhat higher than the normal level.

Our available data for the dairy cow have been classified in Table 8 in two ways: (*a*) according as to whether or not the cow was pregnant when sampled, and (*b*) the values for pregnant cows according to stage of pregnancy.

Table 8. *Serum phosphatase of dairy cows in relation to pregnancy*

Serum phosphatase units	Pregnant		Non-pregnant		Nos. at various stages of pregnancy		
	No.	%	No.	%	0–95 days	96–190 days	191–285 days
0·0– 4·9	3	5·2	1	1·6	1	1	1
5·0– 9·9	10	17·5	27	43·5	2	6	2
10·0–14·9	15	26·3	11	17·7	9	5	2
15·0–19·9	5	8·8	8	12·9	2	1	2
20·0–24·9	3	5·2	4	6·4	2	1	—
25·0–29·9	4	7·0	3	4·8	2	1	1
30·0–39·9	1	1·8	1	1·6	1	—	—
40·0–49·9	9	15·8	1	1·6	1	6	2
50·0–99·9	6	10·5	5	8·0	3	1	1
100·0 and over	1	1·8	1	1·6	—	—	1
Totals	57		62		23	22	12
Under 15 units		49·0		62·8	12	12	5
Under 40 units		71·9		88·8	19	15	9

Comparing the serum phosphatase levels in pregnant and non-pregnant cows in Table 8, it is noted that there is a higher proportion of low values among the non-pregnant cows and that the scatter of values is wider among the pregnant cows, suggesting at first sight a tendency towards higher serum phosphatase values in pregnancy. Our data, therefore, are more in line with those of Vermehren [1939] on women than with those of Wilson & Hart [1932] and Auchinachie & Emslie [1933] on cattle and sheep. It must, however, be admitted that our data are not sufficiently extensive to preclude the possibility of a decrease in serum phosphatase in late pregnancy such as was suggested by the results of Auchinachie & Emslie [1933] on six cows sampled during the fifth and eighth months of pregnancy. The data given in the last three columns of Table 8 do not suggest the existence of any pronounced relation between stage of pregnancy and serum phosphatase level. This, taken in conjunction with the individual records of cows sampled when pregnant and non-pregnant, suggests that the preponderance of low values in non-pregnant cows may be due to chance, though the possibility remains that the physiological condition of pregnancy irrespective of the stage reached is associated with a tendency towards an increase in serum phosphatase.

Serum phosphatase of the dairy cow in relation to milking capacity

In searching for possible causes of the wide individual differences in serum phosphatase of dairy cows it was thought that strain on the Ca and P metabolism due to the large daily excretion of Ca in the milk of high-yielding cows might be reflected in an abnormally high serum phosphatase level [cf. Vermehren, 1939]. Accordingly, available data relating to milk yield and serum phosphatase are presented in Tables 9 and 10.

In Table 9 the cows are classified into two groups, according to whether the yield in the lactation previous to sampling was more or less than 7500 lb.

Though the average lactation yields of the two groups of cows differ widely, the distributions of serum phosphatase values are so similar as to suggest that there is no association between milk yield and serum phosphatase. This conclusion is confirmed by the more detailed data (see Table 10) on 11 Dairy Shorthorn cows of which 5 gave over 9000 lb. and 6 gave under 9000 lb. in the previous lactation.

Table 9. *Average values of serum phosphatase in dairy cows of two different grades of milking capacity*

Serum phosphatase units	Total milk yield in lactation previous to the samplings: Under 7500 lb. No.	Under 7500 lb. %	Over 7500 lb. No.	Over 7500 lb. %
0·0– 4·9	24	29·3	16	26·3
5·0– 9·9	29	35·4	21	34·4
10·0–14·9	9	11·1	8	13·2
15·0–19·9	6	7·3	4	6·5
20·0–24·9	5	6·1	3	4·9
25·0–29·9	1	1·2	2	3·3
30·0–39·9	2	2·4	1	1·6
40·0–49·9	3	3·6	1	1·6
50·0–99·9	3	3·6	5	8·2
Totals	82		61	
Highest yield in lb.	7463		16454	
Lowest yield in lb.	1142		7626	
Average yield in lb.	5830		9224	

Table 10. *Serum phosphatase of 11 Dairy Shorthorn cows of different grades of milking capacity*

Cows giving over 9000 lb. in last lactation: Name	Milk yield lb.	Range of serum phosphatase	Average serum phosphatase	Cows giving under 9000 lb. in last lactation: Name	Milk yield lb.	Range of serum phosphatase	Average serum phosphatase
Carlet 3	13036	53–114	68	Pippin	8864	18–27	24
Peach 3	12358	43– 70	53	Red Rose	8638	8–12	10
Lily 3	9759	10– 17	12	Linda 5	8438	47–68	58
Peach 5	9593	17– 44	26	Patricia	8000	8–13	10
Rosalie	9321	35– 67	44	Campion 2	6868	15–35	23
				Flora 1	4043	8– 9	9
Average	10813		40·6		7142		22·3

Table 11. *Serum phosphatase data of 9 herds representing 6 breeds of cattle*

Serum phosphatase units	Dairy Shorthorn herds: S_1	S_2	S_3	Total	Guernsey herds: G_1	G_2	Total	Other dairy breed herds: D	A	F	Total	Herefords H
0·0– 4·9	—	2	8	10	4	3	7	—	16	26	42	38
5·0– 9·9	20	17	18	55	18	27	45	9	4	4	17	60
10·0–14·9	22	2	9	33	8	14	22	3	—	2	5	12
15·0–19·9	11	2	5	18	2	6	8	1	—	—	1	1
20·0–24·9	10	—	5	15	—	7	7	—	—	—	—	3
25·0–29·9	9	—	6	15	—	1	1	1	—	—	1	3
30·0–39·9	3	2	10	15	—	3	3	2	—	—	2	2
40·0–49·9	10	2	4	16	—	3	3	1	—	—	1	—
50·0–99·9	12	4	5	21	—	1	1	—	—	—	—	—
100·0 and over	2	—	1	3	—	—	—	—	—	—	—	—
Totals	99	31	71	201	32	65	97	17	20	32	69	119

Normal range of serum phosphatase in various breeds of cattle

Data are available (see Table 11) for cows of 6 breeds comprising 3 herds of Dairy Shorthorns (S_1, S_2 and S_3), 2 herds of Guernseys (G_1 and G_2), and 1 herd each of South Devons (D), Ayrshires (A), British Friesians (F) and Herefords (H).

From these data it appears that the serum phosphatase distribution for Dairy Shorthorns differs from that for cows of other breeds in that it is the only breed

exhibiting values over 100 units. Further, about 10% of values for Shorthorns lie between 50 and 100 units compared with only 1% in the case of Guernsey cows and none in the other breeds. This wide scatter of values in the Dairy Shorthorn was first observed in herd S_1, and herds S_2 and S_3 were then studied to find out if the peculiarity was a general characteristic of the breed or merely a peculiarity of the particular herd associated with conditions of management or breeding. Since, however, the distribution was similar in all 3 herds it seems fair to assume that it is a genuine breed characteristic.

Data for Ayrshire and British Friesian cattle are comparatively slender, but it is noteworthy that the values for these breeds are all under 15 units with the great majority of values under 5 units. Intermediate between these breeds and the Dairy Shorthorns come the Guernseys, South Devons and Herefords, the last-mentioned being a 'beef' breed. The herd studied by us was at grass all the year round and kept for calf rearing only. We do not propose to comment further on this breed difference since at present no explanation for it can be offered.

Discussion

One feature of the data which have been summarized in the foregoing tables is of such interest as to merit further discussion. We refer to the surprisingly scattered distribution of the serum phosphatase values in ewes and in cows of the Dairy Shorthorn, and, to a less extent, the Guernsey and South Devon breeds. It is difficult to give any adequate explanation of this phenomenon though one or two possibilities will be considered.

There is a certain amount of evidence which suggests that individual differences in the level of thyroid activity might be responsible for the wide variation in serum phosphatase levels of apparently normal individuals. Kay [1930, 2] found that the serum phosphatase was raised above normal in cases of exophthalmic goitre in man. Scoz & Marangoni [1934] observed a transient rise in plasma phosphatase in one dog after thyroxine treatment. Folley & White [1936] noted an increase in serum phosphatase in lactating cows during thyroxine treatment, but concluded that the changes were not large enough to account for the individual variations in serum phosphatase which had been observed at Shinfield among Dairy Shorthorn cows.

A second possibility is opened up by the observation of Folley [1936] that injection of large doses of oestrogenic hormones caused a temporary rise of the order of 50% in serum phosphatase accompanied by a fall in serum Ca in lactating cows. These changes were interpreted as indicating that oestrogens are capable of exerting an influence on the skeletal system, a contention which has since been repeatedly confirmed [cf. Gardner & Pfeiffer, 1938]. Despite the fact that the doses of oestrogen used in Folley's [1936] experiments were unphysiological and the changes in serum phosphatase not large, the remote possibility remains that individual differences in serum phosphatase might be due in some cases to differences in ovarian activity.

Lastly we would discuss the possibility that the high phosphatase activity of some sera is due to the presence of a phosphatase activator. This possibility was considered by one of us (S. J. F.) but discarded on the basis of evidence acquired in 1935. It was claimed by Thannhauser *et al.* [1937, 1] that ascorbic acid was such an activator, but King & Delory [1938] failed to confirm this and the claim was withdrawn [Thannhauser *et al.* 1938, 1]. In view of the more recent paper of Thannhauser *et al.* [1938, 2] in which the authors claim to have obtained evidence of the presence in sera showing high phosphatase

activity of a substance which, when added to normal sera, causes an increased hydrolysis of phosphoric ester, further discussion of this question seems called for.

In investigating this question at Shinfield in 1935, a serum of relatively high phosphatase activity and two of normal or low activity were used. King & Armstrong [1934] have shown that phosphatase estimations carried out by their method on undiluted sera showing an activity of 60 or more of their units per 100 ml., tend to be low owing to inhibition of the activity of the enzyme by the inorganic phosphate produced by the hydrolysis. Accordingly the high phosphatase serum was diluted with an equal volume of normal saline, this being the normal procedure specified by King & Armstrong for sera showing high phosphatase activity.

The phosphatase activities of the three sera, as well as of mixtures of equal parts of diluted high phosphatase serum with each of the other two, were determined. It was considered that if the highly active serum contained an activator, the activity of a mixture of this with a serum of normal activity would be greater than the arithmetic mean of the activities of the two components. This was essentially the procedure later adopted by Thannhauser *et al.* [1938, 2] to investigate this question. The actual figures obtained are given in Table 12, from which it will be seen that the differences between the theoretical and actual values for the phosphatase activities of the mixtures are well within the limits of experimental error. It would therefore appear that the relatively high phosphatase activity of one of these three sera cannot be ascribed to the presence of an enzyme activator.

Table 12. *Effect of mixing sera of different phosphatase values*

Actual values: serum 1 (diluted 1 : 1), 24·7 units per 100 ml.; serum 2, 9·7 units per 100 ml.; serum 3, 8·9 units per 100 ml.

	Theoretical value	Value found
By mixing equal volumes of 1 and 2	17·2	17·7
By mixing equal volumes of 1 and 3	16·9	17·3
By mixing equal volumes of 2 and 3	9·3	9·2

Thannhauser *et al.* [1938, 2] used a method for the determination of phosphatase activity [see Thannhauser *et al.* 1937, 1] based on the method of Bodansky [1933] but the details of technique are not given fully. In their paper they report that the amount of phosphoric ester hydrolysed by mixtures of equal volumes of normal sera agreed closely with expectation but that when mixtures of normal sera and highly active sera were used the phosphate produced exceeded the expected amount. This was taken as evidence of the presence of a phosphatase activator in the pathological sera. In the absence of full experimental details it is hard to make critical comment on their data, but the fact that the more the highly active serum was diluted with normal serum the more the phospholytic activity of the mixture exceeded the theoretical value, suggests that the true activities of the highly active sera might have been underestimated, on account of the slowing up of the rate of hydrolysis due to the inhibiting action of the end-products, a point guarded against in the technique used by us. Against this possible explanation must be set the statement that when four times as much serum was used, the quantity of ester hydrolysed was proportionately greater, which, if other conditions such as concentration of substrate, total volume of liquid etc. were the same in both cases, would preclude the likelihood of their original determinations on highly active sera having been in error due

to the inhibitory action on the enzyme of the inorganic phosphate liberated. A further point should be borne in mind. The highly active sera of Thannhauser and his colleagues were obtained from cases of Paget's disease in man and from dogs which had undergone ligation of the common bile duct, whereas the results in Table 12 were obtained with sera from clinically normal cows. It is possible that in Paget's disease, or after ligation of the common bile duct, the blood may contain a phosphatase activator which is absent from blood showing high phosphatase activity due to other causes, or that such an activator may be present in highly active sera from man and carnivores but not from ruminants. In support of this possibility it may be mentioned that there is some evidence of species differences as regards changes in serum phosphatase. Thus Thannhauser *et al.* [1937, 2] claim that ligation of the common bile duct causes an increase in serum phosphatase in dogs but not in cats, while rickets is accompanied by an increase in serum phosphatase in man but not in the rat (Prof. H. D. Kay, personal communication). Whatever may be the true explanation of the results of Thannhauser and his colleagues, the postulation of the presence of an activator in highly active sera does not seem to be a likely explanation of the wide range of values encountered among apparently normal cows of the Dairy Shorthorn breed and among ewes.

Summary

1. In apparently normal cows and ewes the variations in serum phosphatase, 0·3–114·3 and 3·0–166·1 units per 100 ml. respectively, are so wide as to lead to the conclusion that its determination can be of little diagnostic significance.

2. Cows of the Dairy Shorthorn breed show a wider range of values and a greater proportion of high values than cows of four other dairy breeds and one beef breed. No explanation of this breed difference can be offered.

3. Though the serum phosphatase may differ widely from animal to animal the general level of the individual remains sensibly constant over long periods of time.

4. In both cattle and sheep serum phosphatase activity progressively decreases with advancing age until maturity is reached. Thereafter cattle show little alteration but there is some evidence in sheep of a further slight decrease with advancing age.

5. There is some evidence that pregnancy *per se* is accompanied by a slightly raised serum phosphatase in dairy cows, though the level of phosphatase does not appear to be correlated with the stage of pregnancy.

6. No evidence was found of correlation between milking capacity and serum phosphatase activity in dairy cows.

7. The high phosphatase activity of the sera of some clinically healthy cows does not appear to be due to an enzyme activator in these sera.

Our best thanks are due to Prof. H. D. Kay and Dr H. H. Green for their supervisory interest in this work. We are also deeply grateful to all those, too numerous to name here, who by their generous co-operation have made it possible to obtain blood samples. Mrs Ruth Allcroft kindly carried out a number of phosphatase determinations, while the technical assistance of Messrs F. J. Salt and S. C. Watson was greatly appreciated.

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27. THE DETERMINATION OF THE VISCOSITY OF HUMAN MILKS AND THE PRENATAL SECRETIONS

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That the marked variations which are known to exist in the viscous properties of human mammary secretions are of interest in studying both normal and pathological conditions has been pointed out by Waller [Waller *et al.* 1941]. In order to assist in a further investigation of the question it seemed advisable to devise a viscosimeter suitable for the study of such materials and to establish the general character of their rheological properties.

In the case of prenatal secretions removed from the breast by manipulation, it is frequently the case that only about a millilitre of material is available for the viscosity measurement, nor is the viscosity always a constant independent of shearing stress. A viscosimeter must therefore be designed which will give a series of readings on a very small sample at a number of different shearing stresses. The instrument shown in Fig. 1 is in principle quite similar to several others already described in the literature, especially by Ostwald and his coworkers, but is designed as the most suitable modification for the present purpose.

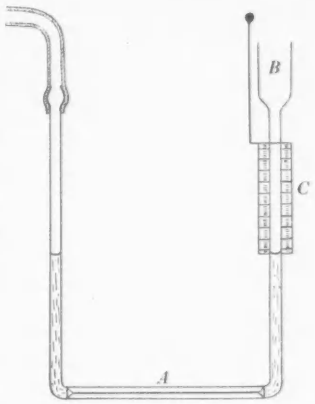

Fig. 1.

The apparatus consists of a glass capillary tube (*A*) sealed into two wider tubes but at right angles so that the whole can be immersed in a thermostat water bath. Approximately 1 ml. of the material to be tested is run by means of a pipette into the wide mouth *B* and allowed to stand for a few moments to reach temperature equilibrium and to find its level on the two sides of the viscosimeter. If there is much 'creaming' this must be done as quickly as possible, the milk being brought approximately to temperature previously in another vessel. A temperature of 37° is used, partly because this is the natural temperature for such materials and partly because it has been shown in the case of cow's milk (especially cream) by Scott Blair *et al.* [1939] that additional rheological complications are introduced when temperatures are used at which the fat phase is liable to solidify.

The sliding scale (*C*) is then adjusted so that the zero of the scale corresponds to the meniscus of the sample.

The sample is then blown into the tube by means of a rubber tube attached to the other arm. When the pressure is released, the column of liquid starts to

fall along the scale (C). When the meniscus passes a convenient point on the scale (h_1) (18 cm. is usually suitable) a stop-watch is started and the time taken for the meniscus to fall to half this height (h_2) is recorded (t_1). The test is then repeated starting at h_2 and timing the fall to h_3, a distance half that of h_2, i.e. a quarter of the original h_1. This second time t_2 should be equal to t_1 if the material is a true fluid obeying Poiseuille's law. If the material is not a true fluid, the ratio of t_1 to t_2 is an index of the extent of the anomaly. The viscosity in C.G.S. units (poises, or better, the kinematic viscosity of which the unit is the stokes, and which is equal to the viscosity/density) is best derived by standardizing the instrument by means of sucrose solutions of known viscosity, and a number of instruments having capillaries of different radii should be used conveniently to cover the whole range of human mammary secretions. A constant is thus obtained for each instrument from which viscosities can be worked out in C.G.S. units (poises or stokes) by multiplying by the value of t_1. Where the viscosities differ for the two ranges of stress, that calculated from a fall from $h_1 = 18{\cdot}0$ to $h_2 = 9{\cdot}0$ is arbitrarily chosen. The errors involved in this choice of stress are small compared with the range of viscosities involved. This simple test takes only a few moments to complete, and the result is calculated by means of a single multiplication.

The above treatment is derived from the following considerations, which also serve to give an absolute standardization of the instrument if such is desired. For materials differing appreciably from truly fluid behaviour, or where only very small samples are available, the direct application of the equation now to be derived is necessary.

Let V be flow rate of sample through capillary in cm.^{3}/sec.,
R_c be radius of capillary (cm.),
P be pressure produced by head h at time t sec., in dynes/cm.^{2},
L be length of capillary tube (cm.),
η be viscosity and ρ the density of sample,
R_w be radius of wide tube (cm.) (assumed large compared with R_c),
v be velocity of meniscus falling in wide tube (cm./sec.).

From Poiseuille's law we have

$$V = \frac{P\pi R_c^4}{8L\eta}$$

(assuming that the viscous resistance in the wide tube is negligible),

or
$$v = \frac{PR_c^2}{8L\eta} \times \frac{R_c^2}{R_w^2} = \frac{PR_c^4}{8L\eta R_w^2}.$$

The rate of decrease of head is twice this, since the meniscus falls on one side but rises at an equal rate on the other; hence

$$\frac{dh}{dt} = -\frac{h\rho g R_c^4}{4L\eta R_w^2},$$

where g is the gravity acceleration,

or
$$t = -\frac{4L\eta R_w^2}{\rho g R_c^4} \int \frac{1}{h}\, dh = -\frac{4L\eta R_w^2}{\rho g R_c^4} \log_e h + C,$$

where C is an integration constant.

Now, when $t = 0$, we have $h = h_0$, hence

$$C = \frac{4L\eta R_w^2}{\rho g R_c^4} \log_e h_0;$$

hence η/ρ, the kinematic viscosity, which we will write ν, is given by

$$\eta/\rho = \nu = \frac{t}{\log_{10}(h_0/h)} \frac{gR_c^4}{4LR_w^2}\, 0{\cdot}4343.$$

Thus, if we are prepared to discount differences in the densities of the samples, i.e. to use kinematic rather than dynamic viscosities, we see that the slope of the curve plotting t against $\log_{10}(h_0/h)$ has only to be multiplied by a constant depending on the dimensions of the apparatus to give the kinematic viscosity in absolute units (stokes). In view of the range of viscosities met with, it is convenient to multiply these figures by 100 and to quote ν in centistokes.

A convenient range of values for R_c is from about 0·03 to 0·08 cm. R_w is conveniently about 0·25 cm. and L about 20 cm.

For most materials the fixing of h_0/h at 2·0 as described in the technique proposed above avoids the trouble of plotting any graphs, but where samples are very meagre, or very anomalous in behaviour, the $t/\log h_0/h$ curve should be plotted. An empirical figure representing the extent of the viscous anomaly is then conveniently obtained by plotting logs of both expressions, the extent to which the slope of the log-log curve differs from unity being a measure of the extent of the viscous anomaly. (For further explanation, see Scott Blair [1938].)

Table 1. *Variation of $t/\log h_0/h$ with time, for water*

t (sec.)	2	4	6	8	10	12	14	16	18	20	22
$t/\log h_0/h$	23·2	21·6	21·3	21·5	22·0	21·8	21·8	21·9	21·8	21·8	21·1

Table 2. *The effect of progressive shearing and resting on $t/\log h_0/h$ for human colostrum*

	t (sec.)	2	4	6	8	10	12	14	15	16	18	20	22	24
Shear No.	1	250	266	286	276	277	279	280	—	271	265	267	275	282
	2	—	235	—	242	—	266	—	—	271	—	278	—	282
	3	—	—	286	—	—	279	—	—	—	272	—	—	289
	4	—	—	—	—	—	—	—	263	—	—	—	—	—
	5*	—	—	—	—	—	—	—	—	—	—	—	—	—
	t (sec.)	26	28	30	32	34	36	38	40	42	44	45	46	48
Shear No.	1	279	—	280	—	278	—	280	—	282	—	—	270	—
	2	—	277	—	278	—	283	—	280	—	286	—	—	282
	3	—	—	297	—	—	290	—	—	282	—	—	—	288
	4	—	—	270	—	—	—	—	—	—	—	281	—	—
	5*	—	—	263	—	—	—	—	—	—	—	—	—	—
	t (sec.)	50	52	54	56	60	64	66	68	72	75	76	78	80
Shear No.	1	274	—	—	—	—	—	—	—	—	—	—	—	—
	2	—	278	—	280	279	281	—	282	287	—	284	—	285
	3	—	—	289	—	290	—	290	—	296	—	—	291	—
	4	—	—	—	—	276	—	—	—	—	281	—	—	—
	5*	—	—	—	—	267	—	—	—	—	—	—	—	—
	t (sec.)	84	88	90	96	102	105	108	114	120	126	132	135	138
Shear No.	1	—	—	—	—	—	—	—	—	—	—	—	—	—
	2	287	292	—	—	—	—	—	—	—	—	—	—	—
	3	291	—	290	292	293	—	294	290	294	294	293	—	295
	4	—	—	283	—	—	281	—	—	285	—	—	289	—
	5*	—	—	278	—	—	—	—	—	281	—	—	—	—
	t(sec.)	144	150	156	165	180	195	210	225	240	255	270	285	300
Shear No.	1	—	—	—	—	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—	—	—	—	—
	3	299	299	294	—	—	—	—	—	—	—	—	—	—
	4	—	291	—	290	294	296	296	297	297	294	294	297	300
	5*	—	283	—	—	290	—	296	—	296	—	311	—	313

* After standing for 1¼ hr., having been gently stirred after ¾ hr.

Experimental

As a check on the accuracy of one of these viscosimeters an experiment was done with pure distilled water. The values of $t/\log h_0/h$ are given in Table 1. They are satisfactorily constant. Some materials show a tendency for the viscosity to fall as a result of repeated shearing [Scott Blair *et al.* 1939]. This makes it hard to decide on any unique value for viscosity. In some cases, the viscosity is partially recovered on standing (thixotropy or false-body). It has been shown (Table 2) that these properties are not present to any marked extent in the case of normal human mammary secretions. Progressive shearing and resting are seen to have little or no effect on this flow curve for a colostral milk sample.

It is not intended to publish here any large number of data or to discuss their bearing on clinical problems. This is being done in another article [Waller *et al.* 1941]. A few typical curves only will be given. These are shown in Fig. 2.

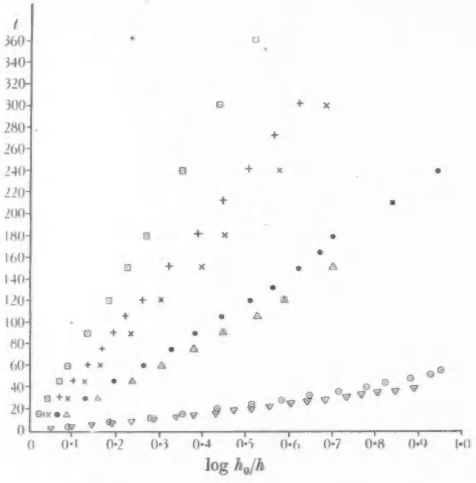

Fig. 2.

	No.	$t/\log h_0/h$	η	Notes
⊙	14	46	1·44	5th day milk, 2nd para.
×	15	394	12·35	38 weeks (about due), 1st para.
△	18	204	6·40	38 weeks, 1st para.
⊡	20	668	20·8	Just after delivery, 1st para.
+	21	470	14·7	3rd day milk, 1st para.
▽	22	42	1·31	4th day milk (engorgement), 1st para
●	23	236	7·38	38 weeks, 1st para.

It will be seen that the curves are very nearly but not quite linear (a few samples showed even more marked anomalies), and that the viscosities varied over a wide range. Although no such extreme samples appear in the figure, values as high as 100 centistokes have been obtained for prenatal secretions, compared with an average of about 1·3 centistokes for normal milks.

The fall in viscosity with rising shearing stress is gradual. There is no sign of a yield value, which leads to the conclusion that all these secretions would

flow through any uncontrolled orifice even at very low pressure. The prevention of seepage thus depends on the behaviour of the ducts rather than on the properties of the secretion even when viscosity is very high.

Summary

1. A method is described by which the viscosity and viscous anomalies of small (1 ml.) samples of human mammary secretions may be quickly and accurately determined.

2. These secretions, tested at blood heat, behave in general surprisingly like true fluids, i.e. their viscosities differ but little with varying shearing stress. The viscosities themselves vary over a very wide range—at least a hundredfold.

3. Where viscous anomalies exist, they appear as a gradual fall in viscosity with rising stress, suggesting that even the most viscous materials would flow slowly under extremely small pressures.

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28. THE VISCOSITY, PROTEIN DISTRIBUTION, AND 'GOLD NUMBER' OF THE ANTENATAL AND POSTNATAL SECRETIONS OF THE HUMAN MAMMARY GLAND

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In his *Clinical Studies in Lactation* Waller [1938] has discussed the manifold problems and difficulties connected with the onset of lactation and has, in particular, drawn attention to the frequency of engorgement with its serious consequences to the maintenance of secretion, and to the possibility of reducing this frequency by the systematic removal of the prenatal breast secretion. It was then pointed out that 'it would be extremely helpful if we knew the relative viscosity of the three types of fluid (prenatal secretion, colostrum and milk) which leave the breast and whether... viscosity bears a direct relation to the protein content'. A suitable method for the measurement of kinematic viscosity was, therefore, developed by Scott Blair [1941], the viscosity of these secretions having so far only been qualitatively assessed [Widdows *et al.* 1935]. It was intended to correlate the viscosity data with the total protein content and, at the same time, to determine some of the major protein fractions, such as globulin and casein. It was, however, soon realized that analytical methods have not been established for the accurate determination of all these constituents in fluids varying so widely in composition as the antenatal and postnatal human secretions. Whilst we were unable to undertake the fundamental investigations obviously needed to provide such methods, we have collected a number of nitrogen partition data of comparative value sufficient to show the approximate levels and changes during the antenatal and early postnatal periods. The viscosity data, however, were correlated with the indisputable total nitrogen figures. In addition, a rapid and simple gold sol reaction was developed which reflects closely the changes in the total nitrogen level of the secretions.

As far as was practicable, samples from each woman included in this investigation were tested on several occasions during pregnancy and daily for the first week or so after parturition. The research thus covers, in part, the same field as that of Lowenfeld *et al.* [1927] and Widdows *et al.* [1935]. It should be pointed out, however, that in the present investigation the prenatal secretion of the subjects was as a rule regularly expressed from the breast. By obtaining results on a relatively large range of material we endeavoured, apart from the establishment of the relationship between viscosity and total nitrogen, to find out whether the regular removal of the prenatal secretion causes marked changes in the physical or chemical make-up of the fluid as it does in the cow [Woodman & Hammond, 1922; 1923; Asdell, 1925]; whether such removal leads to a shortening of the colostral period (for which purpose a number of 'control' cases

was included in which no expression had been practised before childbirth); and whether engorgement exerts an influence on the physical behaviour or the chemical composition of the postnatal secretion.

Methods

With prenatal samples the total volume obtainable by manual expression was used for analysis. With postnatal samples it was not found practicable to apply a uniform method of sampling. Portions of the secretion were obtained either by manual expression or by application of the breast-pump. The samples were taken direct to the laboratory and analysed immediately, or when this was not possible were stored in a refrigerator. Estimations on stored samples at daily intervals showed that no change in viscosity took place during 4 days and that the total nitrogen remained unchanged for 60 hr., though there was a slight rise in non-protein nitrogen.

Storage at room temperature for 3 days did not affect the 'gold number'. This was important, as the samples required for the gold test had to be sent through the post.

Viscosity. The method is described in a separate paper [Scott Blair, 1941].

Nitrogen partition. (*a*) Total nitrogen was determined by micro-Kjeldahl analysis, using 2 ml. of suitably diluted secretions. No standard dilution could be adopted, as the concentration of the samples varied so widely. The viscosity readings provided a useful basis for determining the amount of dilution required in each case.

(*b*) Non-protein nitrogen. Numerous precipitating agents have been recommended for physiological fluids. While giving comparative values, they differ in action, bringing down varying amounts of proteose-peptone compounds in addition to the 'true' proteins. Several of these reagents were tried, but difficulties were encountered with all of them, a number of samples, both ante- and postnatal, giving cloudy filtrates. Pending the development of adequate methods we adopted precipitation with strong trichloroacetic acid, as this reagent seems to provide the nearest approach to the precipitation of the 'true' proteins only [cf. Seibert, 1926].

(*c*) Casein. An adaptation of the method recommended for cow's milk by Rowland [1938] was used. It was found necessary to reduce the amounts of precipitating agents so as to ensure precipitation of the smaller amounts of casein present in human secretions at a *p*H in the neighbourhood of the iso-electric point. Suitable conditions were determined by varying the concentration and proportions of acetic acid and sodium acetate in conjunction with *p*H measurements (glass electrode). The following procedure was adopted for milk: 1 ml. of milk is diluted with *ca.* 15 ml. of water at 40°, 1 ml. of $N/10$ acetic acid is added, followed after 10 min. by 0·8 ml. $N/10$ Na-acetate solution. After cooling, the volume is made up to 20 ml. Rowland's procedure is then followed in detail. The more concentrated prenatal and early postnatal samples were suitably diluted so that the nitrogen content approximated to that of normal milk. Apart from other considerations, this course was necessitated by the fact that often no more than 1 ml. of secretion was available for determining the nitrogen partition.

(*d*) Globulin was determined in the casein-free filtrate as described by Rowland [1938].

'Gold number'

The 'gold number' (Zsigmondy) is defined as the weight of a given protecting colloid just insufficient to prevent a change of colour in 10 ml. of a stable red gold sol when 1 ml. of 10% solution of NaCl is added to it. For the purpose of this investigation the 'gold number' of the ante- and post-natal secretions which contain a mixture of protecting colloids has been defined as the reciprocal of the dilution of secretion contained in 1 ml. of solution which is just insufficient to prevent a change of colour in 5 ml. of a stable red gold sol when 0·5 ml. of 10% solution of NaCl is added to it. It appeared preferable to express the 'gold number' as the reciprocal of the dilution, as in this way a higher 'gold number' corresponds to a higher protecting power of the secretion.

Many workers have encountered difficulties in the routine preparation of gold sols. The technique described by Pennycuick *et al.* [1938] was found to be free from the usual hazards and permitted the preparation of sols of the desired type with ease and regularity. The *p*H of the sols requires no adjustment. Fresh sols should be prepared every 6–8 weeks, as it was noted that after this interval the sols tend to become slightly purplish and to lose their sensitivity.

When dealing with prenatal secretions and early colostrum a series of aqueous dilutions of 1 : 1000, 1 : 2000, 1 : 3000, ... is made up in test tubes. Dilution 1 : 1000 is prepared by diluting 0·1 ml. of the fluid to be tested to 100 ml. using a 0·1 ml. wash-out pipette.

When dealing with later colostrum and milk, dilutions of 1 : 250, 1 : 500, 1 : 750, 1 : 1000, ... are similarly prepared, first making up 1 ml. of fluid (or 0·2 ml. where little material is available) to 100 ml.

1 ml. of each dilution is then pipetted into a wide hard-glass test tube ($5 \times \frac{5}{8}$ in. is a convenient size) and 5 ml. of gold sol are rapidly run in with shaking, followed after 10 min. by 0·5 ml. of a 10% solution of NaCl. The interval between the addition of the gold sol and that of the NaCl solution is not without influence on the result of the test. The period of 10 min. was chosen as being convenient when dealing with several series of samples. Readings are taken after 1 hr., the reciprocal of the first dilution showing a distinct colour change to red-violet being noted as the 'gold number' of the fluid under test. The gold sol in all tubes containing higher concentrations assumes a faint purplish tinge on addition of the NaCl. This uniform change should be disregarded. Duplicate tests were found to agree closely, and with some experience it is often possible to interpolate between the readings of two successive tubes of the series given above. Good agreement was observed when different batches of gold sol were used for the testing of the same dilution series. Cases are encountered in which the 'gold number' drops below 500, and the turbidity of the milk is then apt to interfere to some extent with the colour readings. In such cases useful information is obtained by recording the colour change in the gold sol produced by a 1 : 1000 dilution of the sample.

Results

Viscosity, total nitrogen and gold number

(a) Prenatal secretions

Most specimens were obtained during the last 8–10 weeks of pregnancy. In a few cases it was possible to secure earlier samples; these showed no unusual properties.

The samples varied from a colourless, sticky and apparently fat-free fluid to a rich yellow, creamy mixture from which a thick layer of fat readily separated out on standing.

Space does not permit us to give the results of the serial examinations in detail. They may be summed up as follows: whilst the variations in viscosity, total nitrogen and gold number in individual cases observed at intervals during late pregnancy are considerable, none of these factors shows a systematic decrease or increase as a consequence of the regular removal of the secretion. There is, however, a tendency in many, though not in all, cases for these factors to attain maximum values at the time of, or shortly after, parturition.

The range of values is set out in Tables 1–3, in which only the result of the first examination is included in the many cases in which specimens were repeatedly tested. The tables clearly show the peaks in the frequency of distribution with no particular difference between primiparae and multiparae.

Table 1. *Viscosity of prenatal secretions from 45 women (centistokes)*

	<5	5–15	15–25	>25
27 primiparae	5	17	2	3
18 multiparae	4	11	1	2

Table 2. *Total nitrogen of prenatal secretions from 30 women (g./100 ml.)*

	0–0·5	0·5–1·0	1·0–1·5	1·5–2·0	2·0–2·5	2·5–3·0
18 primiparae	0	3	10	2	3	0
12 multiparae	0	1	9	1	0	1

Table 3. *Gold number of prenatal secretions from 40 women other than those included in Tables 1 and 2*

Gold number	<1000	1000	2000	3000	4000	5000	6000
23 primiparae	0	0	2	12	6	2	1
17 multiparae	1*	0	2	3	6	2	3

* Pathological fluid, patient suffering from severe abscesses.

The gold numbers of specimens from both breasts of the same subject were determined in a number of cases. No marked differences were found, even where differences were noted in the ease of expression, quantity or appearance of the fluids.

(*b*) *Postnatal secretions*

(α) *During the first days of lactation.* The results obtained by following a number of cases through the early stages of lactation proper are summed up in Tables 4–6. They show that the viscosity, total nitrogen and gold number all fall rapidly to new and much lower levels during the first 5 days *post partum*. The initial levels vary greatly, as is indicated by the highest and lowest values given in the tables, but individual cases which cannot be given *in extenso* show a fairly uniform behaviour, namely, a sharp drop from the high to the low level requiring usually between 24 and 48 hr. only. This drop starts in the majority of cases on the 2nd or 3rd day *post partum*, sometimes as early as a few hours after delivery and sometimes as late as the 4th day after parturition.

Lowenfeld *et al.* [1927] followed the protein level in a relatively small number of cases and report a marked difference between primiparae and multiparae, the latter attaining the low level earlier than the former. Examination of the total nitrogen level of a similarly small number of cases which had received no treatment before delivery provided little confirmatory evidence of this observation.

Table 4. *Viscosity of postnatal secretions from 33 women (centistokes)*

Days *post partum*	1*	2	3	4	5	6	7	8	9
	22 primiparae								
Mean	13·95	9·91	5·26	2·07	1·48	1·44	1·34	1·39	1·36
Highest	51·90	25·10	20·00	5·05	1·77	1·80	1·56	1·62	1·66
Lowest	3·11	1·96	1·18	1·24	1·18	1·18	1·13	1·18	1·13

* 7 cases only.

Days *post partum*	1*	2	3	4	5	6	7	8	9
	11 multiparae								
Mean	10·18	14·11	4·51	1·59	1·36	1·46	1·37	1·38	1·32
Highest	23·10	43·00	26·40	1·86	1·58	1·94	1·63	1·84	1·47
Lowest	2·23	2·23	1·56	1·45	1·24	1·24	1·18	1·13	1·18

* 6 cases only.

Table 5. *Total nitrogen of postnatal secretions from 27 cases (g./100 ml.)*

Days *post partum*	1*	2	3	4	5	6	7	8	9
	20 primiparae								
Mean	1·25	1·37	1·42	0·73	0·41	0·37	0·35	0·29	0·33
Highest	1·83	2·00	3·47	1·48	0·67	0·51	0·39	0·43	0·39
Lowest	0·89	0·46	0·32	0·32	0·31	0·28	0·28	0·28	0·29

* 5 cases only.

Days *post partum*	1*	2	3	4	5	6	7	8	9
	7 multiparae								
Mean	1·70	2·02	0·69	0·59	0·34	0·38	0·34	0·31	0·29
Highest	1·71	3·07	0·82	1·24	0·36	0·48	0·38	0·36	0·34
Lowest	1·69	1·39	0·59	0·34	0·31	0·29	0·28	0·27	0·26

* 2 cases only.

Table 6. *Gold number of postnatal secretions from 38 women*

Days *post partum*	1	2	3	4	5	6	7	8
	20 primiparae							
Mean	4000	3500	2000	1200	800	800	800	800
Highest	6000	5000	3500	4000	1000	1500*	1500*	1000
Lowest	2000	1500	750	750	500	500	500	500

* 1 case only, all others 1000 or less.

Days *post partum*	1	2	3	4	5	6	7	8
	18 multiparae							
Mean	4500	4000	1800	1100	800	800	750	800
Highest	6000	8000	3000	1750	1250	1000	1000	1000
Lowest	3000	1750	1250	750	750	500	500	500

No difference was found in the behaviour of untreated cases and of those in which the prenatal fluid had been regularly removed. All cases were, therefore, included in the tables.

As with prenatal secretions, the gold number of samples from both breasts was determined in a number of cases. Here again the results were identical almost without exception, even during the first few days *post partum* with their rapid and drastic changes. Special attention was paid to the incidence of engorgement, which occurred in a more or less severe form in many instances. No evidence was found of any effect of this disturbance on the factors under discussion. The significance of this finding is discussed below.

(β) *During later lactation.* Few cases were followed up regularly beyond the first 10 days or so after parturition, as most patients became unavailable on leaving the hospital. In any case, many data on the total nitrogen level for the ensuing period of lactation are already to be found in the literature [cf. Widdows *et al.* 1930; Bell, 1928], showing that the total nitrogen falls very slowly as lactation progresses. Measurements of the viscosity and the gold number showed that the same applies to these factors. In order to obtain a more sensitive measure of the protecting power under these conditions, the procedure described above of reading the colour of the gold sol in a standard milk dilution of 1 : 1000 was reverted to. All samples obtained between the 5th and 22nd weeks of nursing in cases in which lactation was progressing satisfactorily gave colour changes to blue or bluish violet, whilst the maximum change of colour during the first fortnight *post partum* was one to violet, a reddish violet being the rule.

The relation between viscosity and total nitrogen

By plotting viscosity (ν) against concentration of total nitrogen (c) it was found that these factors are not connected by a simple, linear relationship, the viscosity rising rather more steeply than the concentration of nitrogenous compounds. This is not surprising, considering that Einstein's [1906] equation[1] $\nu = k.c + b$ is based on the assumption of uncharged spherical particles of constant hydration. By plotting log viscosity against the concentration of total nitrogen a straight-line function was obtained, the majority of data fitting the equation

$$\log \nu = 0{\cdot}65.c - 0{\cdot}07$$

reasonably well. Some of the more viscous fluids, however, showed considerable deviations, so that the above equation should not be regarded as more than a convenient generalized expression. No more could be expected considering that with the complex colloidal systems under discussion not only the amount but also the state of dispersion of the colloidal particles will doubtless exert its influence, apart from the possible effect of the fat concentration on the viscosity, which appears, however, to be of secondary importance. That the equation holds in a general way is shown in Table 7, in which the mean values for total nitrogen

Table 7. *Relation between viscosity and total nitrogen*

Days *post partum*	1	2	3	4	5	6	7	8	9
Total N calculated from ν	1·8	1·8	1·2	0·5	0·35	0·35	0·3	0·3	0·3
Total N calculated from Table 5	1·5	1·7	1·05	0·65	0·35	0·4	0·35	0·35	0·3

as calculated from the viscosity data given in Table 4 (primiparae and multiparae combined) are set out against the corresponding total nitrogen values given in Table 5.

The relation between gold number and total nitrogen

Plotting the gold number (G) against corresponding values of total nitrogen concentration (c), a straight-line function was obtained and calculated to

$$G = 3000.c - 200.$$

[1] Einstein's equation was developed for dynamic and not kinematic viscosity, but it seems unlikely that density differences will be great enough to make this distinction significant.

[2] The utility of applying an equation of this type—probably first used by Arrhenius—has been demonstrated by Scott Blair *et al.* [1927], who employed it successfully when dealing with flour suspensions.

With few exceptions this equation was found to hold well within the limits of accuracy of the gold sol reaction. To demonstrate the close agreement obtained the cases in which both the total nitrogen and the gold number were determined simultaneously during the first few days after parturition are summarized in Table 8. The simple gold sol reaction may, therefore, be used to advantage in place of the more tedious Kjeldahl determinations when it is intended to follow the major changes during the prenatal and early postnatal stages of secretion.

Table 8. *Relation between gold number and total nitrogen*

Days *post partum*	2	3	4	5	6	7	8
Total N, mean	1·64	0·81	0·54	0·38	0·35	0·34	0·32
Total N calculated from the gold number	1·54	0·87	0·57	0·36	0·33	0·35	0·33

Nitrogen partition

For the reasons already stated only a limited number of data have been collected.

(*a*) *Non-protein nitrogen and total protein*

Table 9 contains a summary of the results.

Table 9. *Non-protein nitrogen (mg./100 ml.)*

	Mean	Highest	Lowest
15 prenatal secretions	105	200	69
7 postnatal secretions 1–4 days *post partum*	91	127	51
21 postnatal secretions 5–14 days *post partum*	84	140	48

As compared with the total nitrogen, the non-protein nitrogen falls but little during the first few days after parturition. No strictly comparable data are available in the literature. A few values for colostrum and early milk obtained by precipitation with tungstic acid are given by Erickson *et al.* [1934]. Their non-protein nitrogen values are markedly lower and this would seem to indicate the presence of considerable amounts of proteose-peptone compounds in our trichloroacetic acid filtrates. Further investigations in this direction are desirable.

When viewing the non-protein nitrogen data in conjunction with the corresponding total nitrogen figures, it was found that during the prenatal period the non-protein nitrogen constitutes a small and fairly constant proportion of the total nitrogen. During early lactation, however, the percentage of non-protein nitrogen increases markedly, as is shown in Table 10.

Table 10. *Non-protein nitrogen as percentage of total nitrogen*

	%		
No. of cases	0–15	15–30	30–45
Before parturition	15	0	0
1–4 days *post partum*	6	1	0
5–14 days *post partum*	0	15	6

It must be concluded that after the first few days of lactation one is hardly justified in regarding the total nitrogen as a fair representation of the total protein. There is little doubt that the latter continues to fall slowly after 4 days of lactation, whilst the former appears to remain almost constant (see Table 5). This is borne out by the findings of Lowenfeld *et al.* [1927].

(*b*) *Casein nitrogen*

Casein was found to be present in considerable amounts as early as the 22nd week of pregnancy. Up to the time of delivery its concentration changes roughly in proportion to that of total nitrogen; it then shows a similar, if less pronounced, drop during the first 4–5 days of lactation, after which it changes little up to the 14th day *post partum*. The results are summarized in Table 11.

Table 11. *Casein nitrogen* (*mg./100 ml.*)

	Mean	Highest	Lowest
43 prenatal secretions	334	705	100
40 postnatal secretions 1–4 days *post partum*	323	812	114
84 postnatal secretions 5–14 days *post partum*	144	289	64

When the amount of casein is considered as a percentage of the total nitrogen, a definite increase is noted as lactation is established (see Table 12).

Table 12. *Casein nitrogen as percentage of total nitrogen*

	%				
No. of cases	10–20	20–30	30–40	40–50	>50
Before parturition	17	13	12	0	1
1–4 days *post partum*	4	15	15	5	1
5–14 days *post partum*	1	5	27	34	17

(*c*) *Globulin nitrogen*

Globulin was found to be the major protein constituent of the prenatal secretions. During advanced pregnancy its concentration varies, in general, in the same manner as that of total nitrogen. After parturition the globulin continues to behave similarly to the total nitrogen, dropping sharply within the first 4–5 days *post partum*. This drop is, however, followed by a further slow decrease up to 10–12 days after delivery.

The results are summed up in Tables 13 and 14.

Table 13. *Globulin nitrogen* (*mg./100 ml.*)

	Mean	Highest	Lowest
41 prenatal secretions	825	1896	281
39 postnatal secretions 1–4 days *post partum*	556	2080	66
83 postnatal secretions 5–14 days *post partum*	78	214	33

Table 14. *Globulin nitrogen as percentage of total nitrogen*

	%	
No. of cases	0–40	40–80
Before parturition	2	39
1–4 days *post partum*	13	26
5–14 days *post partum*	80*	3

* Mostly 10–30%.

(*d*) *The ratio globulin/casein*

Perhaps the most characteristic difference between the antenatal and postnatal secretions is seen in the ratio globulin/casein as set out in Table 15.

Table 15. *Globulin/casein ratio*

No. of samples	Ratio >1·0	Ratio <1·0
Before parturition	37	2
1 day *post partum*	4	1
2 days *post partum*	8	0
3 days *post partum*	9	1
4 days *post partum*	6	7
5–14 days *post partum*	7	76

Discussion

Woodman & Hammond [1922; 1923] and Asdell [1925] have shown that the regular removal of the prenatal secretion of the cow or heifer causes drastic changes in the physical and chemical properties of the fluid as pregnancy progresses and leads to a state simulating lactation proper. The results presented here indicate that in the human subject the effects of such removal are fundamentally different. The protein make-up and the viscosity of the fluid, though subject to variations, do not alter systematically and there is nothing to indicate that regular expression stimulates the production of an increased amount of a more milk-like fluid. It seems significant that in the human subject the ratio globulin/casein remains high until some time after parturition, whereas in the case of the heifer studied by Asdell this ratio fell below 1·0 as early as the 32nd week of pregnancy.

In agreement with Widdows *et al.* [1935] we found that the prenatal secretions fell into three distinct classes, the appearance of each being unconnected with the state or number of pregnancies. Our attempts to gauge the viscosity from the appearance of the secretion in the manner described by Widdows *et al.* failed, the result of the actual measurement being often in disagreement with the visual judgement. That these authors were, nevertheless, able to predict a definite relation between the viscosity and the protein content of the secretions may be explained by the fact, brought out in our investigations, that small variations in the protein content correspond to large changes in viscosity, the total nitrogen (and, therefore, presumably the total protein) being connected by a linear function with the logarithm of viscosity and not with the viscosity itself.

Turning to the postnatal stages, it seems necessary, first of all, to remark briefly upon the term 'human colostrum'. The uncertainties attaching to this term have been fully discussed by Lowenfeld & Widdows [1928] and by Waller [1938]. Here it suffices to point out that whilst it is true that the human secretion continues to differ in some respects from 'mature' milk up to the end of the first month of lactation, a fluid essentially similar to this 'mature' milk is already being secreted after a few days of lactation only. Is it unreasonable to suggest that the term 'colostrum' should not be applied to this milk-like fluid, but that its use should rather be confined to the fluid fundamentally different from milk which is produced during the first few days after parturition? The results presented above seem to us to favour this suggestion. They show that measurable criteria of both a physical and a chemical nature are available, viz. the viscosity, the total nitrogen content and the globulin/casein ratio, all pointing to the conclusion that the time during which 'colostrum' is secreted is short and does not, as a rule, exceed a period of more than 5 days after delivery. Of these criteria the viscosity and the total nitrogen, or—in its place—the gold number, lend themselves most readily to measurement, but the ratio globulin/casein is perhaps

of greatest significance, as it reflects a fundamental change from the predominance of globulin to that of casein. It is to be hoped that more satisfactory methods for the determination of these protein fractions will soon become available.

Contrary to Lowenfeld *et al.* [1927], we found no difference between the duration of the colostral stage in primiparae and multiparae in untreated cases. Neither was there any evidence that the regular removal of the antenatal secretion shortens the colostral period, and this fact, taken in conjunction with the observations made on the prenatal fluids, speaks strongly against the conception of stimulation of the lactiferous glands by this treatment.

It has been shown that the transition from colostrum to 'early milk' does not, in general, require more than 24–48 hr. It seems likely that the starting time depends on the time required by the body to set in motion the hormonal functions controlling lactation proper and that, once these have begun to work, milk rapidly replaces colostrum. If to this is added the observation that engorgement has no demonstrable effect on the physical or chemical nature of the fluid within the breast, one is driven to the conclusion that the mechanism of milk production is established with truly remarkable regularity and that it is the mechanism of expulsion rather which is so frequently at fault. It follows that, contrary to expectations at the outset of this investigation, a study of the composition and properties of the secretions cannot be of help in explaining the beneficial effect of the prenatal treatment which has been established beyond doubt by clinical observations at the British Hospital for Mothers and Babies. The causes of engorgement must be sought rather in factors connected with the mechanism of outflow. Whether the prevention of engorgement by prenatal treatment is due to a simple 'training' effect or to specific anatomical, physiological or psychological factors remains to be seen. As engorgement presents itself as the consequence of 'a gland functioning actively against obstruction to outflow' (Waller), future investigations must clearly be directed towards elucidation of the nature of the hindrance. A promising line of approach is suggested by the interesting study of Seitz [1924–25], whose work on the relation between structure and function of the breast provides ample evidence of the frequency with which hindrance to outflow is caused by anatomical factors.

Summary

Data are presented showing the level of, and changes in, viscosity, total nitrogen, globulin and casein in human mammary secretions during late pregnancy and early lactation. In addition a gold sol reaction is described which permits the rapid determination of a factor, the 'gold number', shown to be linearly connected with the total nitrogen content of the secretions.

The results show that regular removal of the antenatal fluid does not lead to systematic alterations in composition. Characteristic changes of the factors investigated occur during the first few days *post partum*, and it is proposed to confine the use of the term 'colostrum' to the fluid secreted during this period.

The data are interpreted as suggesting faults in the mechanism of expulsion rather than faults in the mechanism of milk production as the principal cause of the lactational difficulties encountered so frequently during the early postnatal stages.

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29. THE COLORIMETRIC ESTIMATION OF TOTAL CHOLESTEROL IN WHOLE BLOOD, SERUM, PLASMA AND OTHER BIOLOGICAL MATERIAL

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Since Autenrienth & Funk [1913] and Weston [1917] first drew attention to its inaccuracies, the colorimetric method for the estimation of cholesterol based on the Liebermann-Burchard reaction [Grigaut, 1910] has undergone many modifications. It has been the author's experience, however, that no method yet published has succeeded in entirely overcoming the difficulties mentioned by these authors.

With the majority of modern photoelectric colorimeters the colour produced in the test solution alone is measured and the required result is obtained by consulting a previously drawn calibration chart, not by comparing the colour with that of a standard simultaneously developed. It is essential therefore that the colour be exactly reproducible. With this in mind a study was made of existing methods of colour development [Bloor, 1913–14; 1914; 1915; 1916; 1928; Autenrienth & Funk, 1913; Cornell, 1928; Schoenheimer & Sperry, 1934; Reinhold & Sheils, 1936], with a view to adapting them for use with the photoelectric colorimeter. Using a standard solution of cholesterol in chloroform, not one of the above methods gave satisfactorily reproducible results, and furthermore, when the standard solution was replaced by a cholesterol solution prepared from blood by existing methods of extraction [Bloor, 1913–14; Sackett, 1925; Myers & Wardell, 1918; Reinhold & Sheils, 1936], the variations were accentuated in all cases.

The object of this investigation was therefore to examine the factors influencing the accuracy of the colorimetric method for the estimation of cholesterol and to utilize the results obtained to devise a more accurate method.

The investigation fell into two sections: the study of (*a*) methods of extraction of cholesterol from blood; (*b*) methods for the development of the colour.

Extraction methods. Two general methods exist for the extraction of cholesterol: (1) chloroform extraction of blood dried by heat or by dehydrating agents [Myers & Wardell, 1918; De Toni, 1926; Leiboff, 1924; Ling, 1928; Reinhold & Sheils, 1936]; (2) extraction from fresh blood by means of alcohol, alcohol-ether, acetone etc. These latter methods are generally accepted as being more complete, and that due to Bloor [1913–14] is the one most frequently used. Their great disadvantage lies in the fact that the extract has a yellow brown colour which leads to high and erratic results [Mueller, 1916; Weston, 1917; Luden, 1917]. Various methods have been suggested for the elimination of this interference. Mueller [1916] washed the chloroform extract with water and then dried it with anhydrous sodium sulphate. Stoddart & Drury [1929] and others have advocated saponification before solution in chloroform. Sackett [1925] claimed that the colour

could be avoided if the ether-alcohol extraction was carried out without heating. Cantoni [1930] suggested adding a mixture of oleic and palmitic acids to the standard to compensate for the colour. Bloor [1928] advised the use of a red glass filter in the colorimeter when there was any serious degree of discoloration. Schoenheimer & Sperry [1934] and Kelsey [1939] avoid this difficulty by saponifying the extract and removing the cholesterol as digitonide, which is then purified and analysed.

Methods for the development of the colour. The following are the conditions laid down in existing methods for the control of the development of the green colour produced by the interaction of acetic anhydride and sulphuric acid with cholesterol in chloroform solution:

Bloor [1913–14; 1928]. Stand for 15 min. in the dark or in the light in which the comparison is to be made—no temperature given.

Autenrienth & Funk [1913]. 15 min. at 35°.

Weston [1917]. Add H_2SO_4, mix, stand in dark 30 min., then add the acetic anhydride and stand 15 min.

Cornell [1928]. 15 min. away from light at laboratory temperature.

Schoenheimer & Sperry [1934]. Warm solution to 25° before adding reagents, develop colour in water bath at 25° and read between 27–37 min. The amount of H_2SO_4 added is described as 0·1 ml. (or 4 uniform drops).

Reinhold & Sheils [1936]. 30 min. at 25° in the same light in which the comparison is to be made. The solution is not previously heated but warm acetic anhydride-sulphuric acid mixture is added.

Kelsey [1939]. Set in water bath at 23° for 15 min. under moderate conditions of lighting.

The factors concerned are therefore: light, concentration of reagents and of cholesterol itself, temperature and time, and one other not mentioned above, namely, the condition of the cholesterol whether free or esterified. Some of the above methods use extracts prepared directly from blood and therefore containing both esterified and free cholesterol, others use saponified extracts containing only free cholesterol. Gardner & Williams [1921] found that esterified cholesterol reacted at a greater rate than free cholesterol and that cholesteryl acetate gave an 'apparent cholesterol' 10% greater than an equivalent amount of free cholesterol; cholesteryl benzoate gave values 4·6% greater than free cholesterol. Kelsey [1939] found that the colour produced by cholesteryl palmitate was 130% of that produced by an equivalent amount of free cholesterol. Reinhold & Sheils [1936], using their method of colour development, confirmed the findings of Gardner & Williams [1921] and reported an 'apparent cholesterol' for cholesteryl palmitate of 120%. Yasuda [1936] has reported the palmitate as giving 24·5%, the stearate 25·2% and the oleate 27·6% greater values than an equivalent amount of free cholesterol.

Experimental

The factors controlling the development of the colour were studied first in order to try to evolve a standard method which could be used to compare the various extraction methods. Each factor was investigated in turn while the others were kept as constant as possible.

Light. It was found that light had a marked effect on the colour development, as will be seen in the following experiment, in which two series of exactly similar solutions of cholesterol were treated alike in every respect except that the colour development in one series took place in the dark and in the other in the light.

	Colour developed in light Absorption density 640 mμ	Colour developed in dark Absorption density 640 mμ
1	0·357	0·387
2	0·362	0·387
3	0·355	0·387
4	0·333	0·389
5	0·352	0·382

The series developed in the light, it will be seen, gave paler solutions which varied in intensity, due probably to unequal lighting. Hence in all subsequent experiments the colour was allowed to develop in the dark.

Concentration of reagents. Alteration of the concentration of the reagents, particularly that of H_2SO_4, gave rise to changes in the final colour.

	ml.	Absorption density 640 mμ
Solution A	4·7 standard cholesterol solution 0·2 chloroform 0·1 conc. H_2SO_4 2·0 acetic anhydride	0·387
Solution B	4·7 standard cholesterol solution 0·1 chloroform 0·2 conc. H_2SO_4 2·0 acetic anhydride	0·373
Solution C	4·7 standard cholesterol solution 0·3 conc. H_2SO_4 2·0 acetic anhydride	0·367

This difficulty can only be overcome by standardizing the amounts of reagents present. In this investigation 2 ml. of a mixture, prepared not more than 10 min. previously, of acetic anhydride A.R. (20 ml.) and conc. H_2SO_4 A.R. (1 ml.) were added to every 5 ml. of chloroform solution of cholesterol.

The concentration of cholesterol itself is another factor to be considered. Methods quoted above use concentrations varying from 3·0 to 0·2 mg. per 5 ml. chloroform (i.e. amounts roughly equivalent to those present in 1·5–0·1 ml. of normal serum). Concentrations of 0·2–0·6 mg. per 5 ml. were taken in these experiments, largely because the depth of colour produced was convenient for the photoelectric colorimeter used. Later experiments showed that with higher concentrations of cholesterol the colour formation tended to become erratic.

The two remaining factors temperature and time were considered together. Fig. 1 shows the relation between absorption density and time at 18°, 25° and 35°, all other factors being standardized.

Each point was determined under the following conditions. To 5 ml. of a standard solution (=0·289 mg.) of cholesterol in chloroform at 18°, 25° or 35° were added 2 ml. of a mixture of acetic anhydride and conc. H_2SO_4 (20 : 1) freshly prepared and warmed to the same temperature. After careful mixing the solution was placed in a beaker of water maintained at the required temperature in the dark and removed at the expiration of the required time. The same tubes were used throughout, being as far as possible of the same thickness, to prevent irregularities of heating. The readings were taken on a Bolton-Williams type of photoelectric colorimeter, using as the blank 5 ml. of chloroform with 2 ml. of acetic anhydride-sulphuric acid mixture which had been treated in the same way as in the test. The absorption of light (640 mμ) by a

layer of solution 1 cm. in thickness was measured and the result was converted into absorption density (which is proportional to cholesterol content).

It will be seen from the curve that the colour is partially destroyed by heat, the maximum point attained decreasing with temperature. The curve for 35° shows that this temperature is obviously unsuitable for colorimetric purposes, the changes of colour being too fast to give adequate time for a reading to be made.

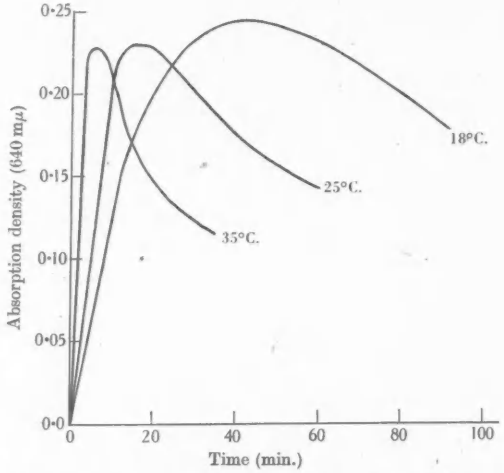

Fig. 1. Colour development charts for free cholesterol at 18°, 25° and 35°.

The same may be said with less force of the 25° curve, the rate of change corresponding to as much as 2·1 mg./100 ml. in the original blood per min. at 30 min.

Further, 25° is a difficult temperature to maintain exactly in a small laboratory without special apparatus for the purpose. Small differences in heating due to outside factors such as differences in the thickness of the tubes cause appreciable variations. At 25° and 30 min. twenty-five estimations gave: average absorption density 0·209 (145) $\pm$ 0·015; max. 0·223 (155) and min. 0·190 (131). (The figures in brackets give the approximate equivalent in terms of mg./100 ml. normal serum.)

The optimum temperature proved to be 18° and the time 60 min., the fading of the colour being sufficiently slow to permit an accurate reading to be made, even when a number of estimations are being carried out at the same time. It was also quite easy to maintain this temperature. Results, using these conditions, were exactly reproducible. Twenty-five estimations on the same solution gave an average absorption density 0·234 (145) $\pm$ 0·002 with max. 0·236 (147) and min. 0·232 (143).

Another factor not yet mentioned is the presence of water in the reagents, particularly the chloroform. Previous methods have insisted on the use of water-free reagents without giving any explanation for this precaution. This is really only another demonstration of the influence of temperature on the reaction. The heat evolved by the addition of conc. H_2SO_4 to chloroform containing only traces of water is sufficient to accelerate the colour development and the subsequent fading and hence lower the final result. All reagents should therefore be completely water-free.

Condition of the cholesterol. The last factor, namely, the state of the cholesterol, was investigated as follows. In each of four 100 ml. conical flasks were placed 30 ml. of chloroform containing 1·794 mg. of cholesterol as (1) free cholesterol, (2) cholesteryl acetate, (3) cholesteryl oleate, (4) cholesteryl stearate. The four flasks were then placed in the dark in a water bath maintained at 18°. To each were added 12 ml. of a freshly prepared mixture of acetic anhydride and conc. H_2SO_4 cooled to 18°. The absorption of light of wave-length 640 mμ by each of the solutions was measured at frequent intervals and a chart prepared showing the relation between time and absorption density (colour) of the solutions (Fig. 2).

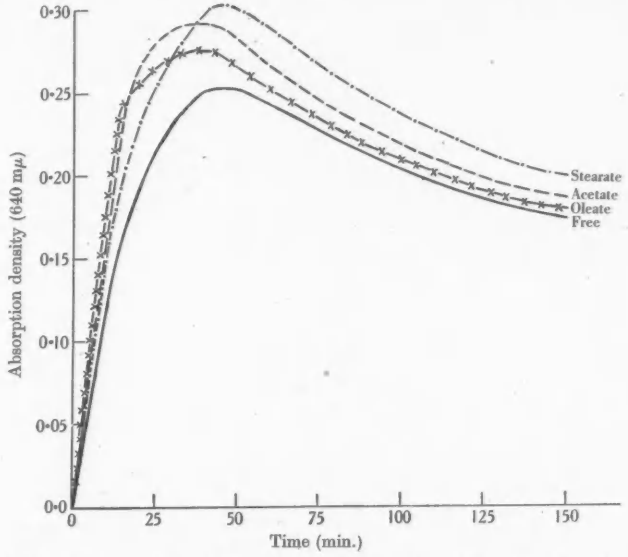

Fig. 2. Colour development charts for cholesterol and its esters (18°). Key: Cholesterol ——. Cholesteryl oleate ×—×. Cholesteryl acetate — — —. Cholesteryl stearate —·—.

The following points are worthy of note.

(1) The four curves are similar in shape.

(2) All esters show a faster colour development, a deeper maximum colour, and a faster colour fading than free cholesterol.

(3) The difference between cholesterol and its esters is greatest in the region of maximum colour development and diminishes as the colours fade, tending to become constant; readings taken after 24 hr. still showed higher values for esters than free cholesterol.

(4) Each ester has its own characteristic rate of colour development and also maximum colour. After a certain time the rate of colour fading is approximately the same in all cases.

(5) This experiment was repeated at various temperatures, but no conditions could be found in which the same amount of cholesterol gave an identical colour in all four forms.

The above facts explain the great diversity in the 'apparent' cholesterol values obtained by previous workers for the various esters. Using the conditions

specified above, the 'apparent' cholesterol values for cholesteryl stearate, oleate and acetate are respectively 119, 103·2 and 109·2 % of that for free cholesterol.

The obvious method of eliminating this difference between the various forms is to convert them into one common form; saponification presents the easiest way of doing this. Difficulties were encountered in adapting existing methods. To test them, two chloroform solutions were prepared, each containing the same weight of cholesterol per unit volume, one as free cholesterol, the other as cholesteryl stearate. 5 ml. portions of the cholesteryl stearate solution were taken and after removal of chloroform subjected to the various procedures suggested for the saponification of blood fats; they were then redissolved in 5 ml. chloroform. The 'apparent' cholesterol content of the saponified extracts and also that of 5 ml. of the original cholesteryl stearate solution were determined colorimetrically (using the procedure developed above) by comparison with the colour simultaneously developed by 5 ml. free cholesterol solution.

The following are some typical results taken at random from a large number of analyses to illustrate the type of results obtained.

Material	Method of saponification	'Apparent' cholesterol mg. (*a*)	(*b*)	Remarks
0·386 mg. free cholesterol	None	0·386	0·386	Blank
0·652 mg. cholesteryl stearate	None	0·463	0·463	Actual cholesteryl content = 0·386 mg.
0·386 mg. free cholesterol	Aqueous KOH (10 %)	0·386	0·386	Test of extraction by chloroform (*v. infra*)
,,	,,	0·384	0·386	
0·653 mg. cholesteryl stearate	Aqueous KOH (10 %), 3 hr. in sealed flask	0·415	0·422	Chloroform extraction. Extract colourless
0·652 mg. cholesteryl stearate	1·0 *N* sodium ethylate [Bloor, 1928]	0·360	0·370	Light petroleum extraction. Extract yellow
,,	,,	0·410	0·418	Chloroform extraction. Extract yellow
,,	Alcoholic (70 %) KOH (10 %), 3 hr. at 40°, alcohol evaporated off at 70°	0·431	0·423	Chloroform extraction. Extract yellow
,,	Alcohol-acetone KOH (10 %), 3 hr. at 40°, alcohol-acetone evaporated off at 70°	0·425	0·440	Chloroform extraction. Extract yellow
,,	2 ml. aqueous KOH (10 %), 1 ml. amyl alcohol, 90 min. at 100° in stoppered flask	0·386	0·386	Chloroform extraction. Extract colourless

A serious difficulty encountered here was the fact that in the majority of the methods, after saponification the cholesterol is not extracted from the aqueous-alcohol media as such but is precipitated as cholesteryl digitonide. Early experiments after the method of Bloor [1928], using light petroleum as solvent, gave rather low results (see table). Light petroleum, while being a good general fat solvent, is not a sufficiently good one for cholesterol. Attempts were therefore made to extract cholesterol quantitatively from strong aqueous alkaline solutions by means of chloroform, which is easily the best cholesterol solvent. As will be seen from the table, 100 % recovery was obtained of cholesterol added to 2 ml. portions of a 10 % aqueous solution of KOH. Details of the actual technique used are given later in the section on method.

The general conclusion to be drawn from the above table is that alcohol is unsuitable as a solvent during saponification for colorimetric analysis, since,

wherever it is used, the final chloroform extract is tinted yellow, and since this colour is increased by heat it is difficult to apply sufficient heat to the system to keep the cholesterol in solution and to obtain complete saponification. Amyl alcohol, on the other hand, although less miscible with water has the advantage that it is less volatile and no colour is produced during saponification. 2 ml. of 10% aqueous KOH and 1 ml. of amyl alcohol at 100° in a closed flask for 90 min. completely saponified 0·652 mg. of cholesterol stearate. Complete saponification of cholesteryl acetate and oleate was also obtained by this same method. The application of the method to cholesterol-containing extracts prepared from blood will be considered after the next section.

Extraction methods. Only the standardized method of colour development evolved above was used in the analysis of the various extracts referred to in this section.

All methods involving the drying of blood gave consistently low results and poor recovery of added cholesterol. It was found difficult, if not impossible, to obtain complete extraction of cholesterol under these conditions, a conclusion agreeing with that of other workers in this field.

As regards 'wet' methods, the yellow colour of the extract already referred to presents a big difficulty. Below are some of the results obtained in experiments carried out to test the suggestions quoted earlier for removing it.

The colours of chloroform extracts prepared from the same serum by the various methods were compared in terms of their absorption of light of wavelength 440 mμ.

Extraction method	Method of purification	Percentage absorption of light, 440 mμ (*a*)	(*b*)
Hot ether-alcohol	None	9·0	9·5
Hot ether-alcohol	Mueller's method	9·0	9·0
Hot ether-alcohol	Saponification (sodium ethylate)	11·0	12·5
Cold ether-alcohol	None	7·0	6·5

The remaining methods were found to be equally ineffective in overcoming the difficulty. It will be noted that results from Sackett's method indicate that heating in Bloor's original method increases the colour, as also does saponification with sodium ethylate.

Theoretically the procedure advocated by Schoenheimer & Sperry [1934] and Kelsey [1939], viz. saponification of extract, removal of cholesterol as digitonide, its saponification and finally its analysis, is more accurate, but it is open to criticism from a number of points of view. For clinical purposes it is too long and complicated and the number of stages involved tends to increase the possibility of error. In practice it was found difficult to isolate quantitatively so small an amount of the very light particles of cholesteryl digitonide merely by centrifuging them. The method of colour development is also open to criticism; in addition to the points already noted, i.e. time, temperature, concentration of reagents etc., it was noted that the digitonin present in the solution after the liberation of the cholesterol from the digitonide itself gave a reddish-purple colour with the acetic anhydride-sulphuric acid mixture. To compensate for this Schoenheimer & Sperry advocated the use of colour filters. My experiments on these lines indicated that whatever colour screen was used some light absorption could always be detected.

It was therefore decided to revert to Bloor's original ether-alcohol extract and endeavour to find some method whereby it could be decolorized without loss of cholesterol. This was finally accomplished as follows. When a yellow-brown chloroform extract prepared from blood by Bloor's method was passed through

a column of finely divided silica gel, the issuing fluid was found to be completely colourless. When solutions of pure cholesterol were passed through, followed by chloroform washings, 100% recovery of cholesterol was obtained in every case.

The following are some typical results.

	Optical colorimeter			Photoelectric colorimeter		
	A	B	C	A	B	C
Bloor's extract untreated, coloured	205	196	220	194	190	216
Ditto after silica treatment, colourless	190	187	210	190	188	212
Ditto plus added cholesterol equivalent to 50 mg./100 ml. after silica treatment, colourless	240	237	260	240	238	262

As would be expected results are lower after the removal of the interfering colour. The fact that the difference is less marked in the case of the photoelectric colorimeter can be accounted for as follows. The green colour was measured in terms of its percentage absorption of light of wave-length 640 mμ (red brown); the percentage absorption of this wave-length by the similarly coloured solutions before colour development averaged only 0·8%, which is equivalent to an apparent cholesterol of 4 mg./100 ml. original serum. This, it will be seen, corresponds with the average difference between the results before and after silica treatment. The brown pigment is apparently not affected by the sulphuric acid-acetic anhydride mixture [cf. Luden, 1917] and therefore in cases where extreme accuracy is not required it is justifiable, in the case of serum or plasma, to omit the silica treatment provided that the colour is measured in terms of its percentage absorption or transmission of light of wave-length 640 mμ. When estimating the cholesterol content of whole blood or red blood cells the interfering colour is much stronger and should always be removed.

The practical details of the silica treatment are given later in the section on method. In addition to blood the method has proved of even greater value in tissue analysis, since the brown colour which is often present in larger amounts in tissue extracts is completely removed by the silica.

Application of saponification method to blood extracts. The cholesterol content of a number of 2 ml. samples of whole blood and serum was determined by the following procedure: extracting with ether-alcohol, filtering, evaporating off the ether-alcohol, extracting the residue with chloroform, decolorizing the chloroform extract, removing the chloroform, saponifying and finally extracting the cholesterol for colorimetric analysis, according to the conditions already noted. Simultaneously, further ether-alcohol extracts of 2 ml. samples of the same blood and serum were prepared and to these were added known amounts of cholesterol or cholesterol ester. The total cholesterol content was then measured in the same manner as above. The results (below) showed a good recovery of added cholesterol, indicating that the figures for whole blood and serum were also consistent.

	Serum A, mg. cholesterol		Serum B, mg. cholesterol		Blood C, mg. cholesterol		Blood D, mg. cholesterol	
Material	1	2	1	2	1	2	1	2
0·2 ml. blood or serum	0·305	—	0·352	—	0·210	—	0·242	—
0·2 ml. blood or serum + 0·193 mg. free cholesterol	0·500	0·195	0·545	0·193	0·404	0·194	0·435	0·193
0·2 ml. blood or serum + 0·193 mg. cholesterol as stearate	0·498	0·193	0·545	0·193	0·405	0·195	0·435	0·193
0·2 ml. blood or serum + 0·193 mg. cholesterol as oleate	0·498	0·193	0·545	0·193	0·403	0·193	0·432	0·190

The figures in the second columns give the actual cholesterol recovery.

Method

Preparation of ether-alcohol extract

2 ml. of whole blood, serum or plasma are run slowly into 60 ml. of freshly mixed ether-alcohol (3/2) in a 100 ml. conical flask, the liquid being gently shaken with a swirling motion. The flask is then immersed in hot (80°) water and rotated continuously to prevent superheating. When the contents of the flask have been brought to the boil it is removed and cooled in running water to room temperature. The solution is next filtered into a dry graduated 100 ml. flask, the conical flask and the residue on the paper being washed several times with further quantities of warm ether-alcohol mixture. When cool the combined filtrates are made up to 100 ml. and well mixed.

Removal of colour

50 ml. of this solution are transferred to a 100 ml. squat-form beaker and evaporated just to dryness on a water bath. The residue is then extracted three times, twice with 5 ml. and once with 3 ml. of warm dry chloroform, the extracts being decanted into a small clean dry separating funnel.

A silica adsorption tube is meanwhile prepared as follows. A clean dry test tube $6 \times \frac{1}{2}$ in. is heated in the bunsen flame about 1 in. from the sealed end and drawn out to a capillary (about $\frac{1}{12}$ in. bore) which is cut 1 in. from the main part of the tube. When cool, a lightly packed plug of cotton wool is placed in the tube at the point where it begins to narrow, and the tube is filled to within $\frac{3}{4}$ in. of the top with finely divided pure silica (see below). A second plug of cotton wool is placed on top of the silica. The tube is fitted by means of a rubber stopper into the neck of a side-arm test tube (30 ml. capacity), or small filter flask, which is connected to the filter pump. 5 ml. of dry chloroform are allowed to flow through the adsorption tube and the residue is removed by suction. The receiver is changed and first the chloroform extract and then 15 ml. of pure dry chloroform are allowed to run through (without suction) at the rate of 15–20 drops per min.; the final portion of the chloroform is removed from the silica by turning on the filter pump, slowly at first, then at full pressure, for 5 min. The chloroform extract and washings are completely transferred to a dry 50 ml. graduated flask and made up to volume; the flask is stoppered and well shaken to ensure thorough mixing.

Saponification of esters

10 ml. of chloroform solution are pipetted into a clean dry 120 ml. pyrex conical flask fitted with a ground glass stopper. The flask, unstoppered, is placed in a steamer (if a bacteriological one is not available one of the household kind can be made to serve the purpose), until all the chloroform has been removed. 1 ml. of amyl alcohol is then pipetted into the flask, followed by 2 ml. of 10% aqueous KOH. After its contents have been well mixed by shaking, the flask is replaced in the steamer. Twenty minutes later the stopper is placed in position and the heating continued for 90 min.: from time to time the flask, without being removed from the steamer, is given a swirling motion to agitate its contents. (Care should be taken that the stopper is not blown out.) At the end of the required time the flask is removed from the steamer and the stopper withdrawn while still hot. When the contents of the flask have cooled to 40°, 5 ml. of chloroform are run in and the flask is then shaken with a vigorous swirling motion, care being taken not to spill any of the contents. After allowing a few

minutes for it to settle, the chloroform-water mixture is decanted into a clean dry test tube (thick walled, 30 ml. capacity), the last few drops being washed in with the aid of a chloroform wash bottle. The stoppered flask is then carefully washed out with two 3 ml. portions of warm chloroform, the washings being added to the main chloroform-water mixture in the tube. The test tube is filled to within $\frac{3}{4}$ in. from the top of the tube with distilled water, tightly stoppered and inverted several times. When the two layers have separated, the tube is given a number of sharp taps at about the level of the water meniscus to dislodge the small drops of chloroform that usually remain in the meniscus, and it is then replaced in the rack for a further 3 min. Having made sure that all the chloroform has settled into the lower layer, the bulk of the upper layer is drawn off by means of gentle suction, using a narrow glass tube drawn to a jet, the tip of which is turned upwards. An aqueous layer about $\frac{1}{8}$ in. deep is left above the chloroform and to this are added 10–12 ml. of N HCl; the tube is then stoppered and inverted several times and the contents are allowed to settle as before. The HCl washing is repeated twice, followed by three water washings in the same manner. After the final water washings have been removed the chloroform extract is decanted into a clean dry 50 ml. beaker and the tube is washed out twice with 2 ml. portions of chloroform, the washings being added to the main bulk of the solution. The beaker is then placed on a hot water bath at 80° and the extract evaporated just to dryness. The residue is dissolved by warming with 2 ml. of pure dry chloroform, the solution is decanted with the aid of a glass rod and chloroform wash bottle into a small test tube ($5 \times \frac{1}{2}$ in.) graduated at the 5 ml. mark. The beaker is washed out with further portions of chloroform and when cold the combined extracts are made up to 5 ml. with dry chloroform.

Development of colour

The tube is corked and placed in a water bath maintained at 18° and side by side with it is placed a test tube containing a freshly prepared mixture of 20 : 1 acetic anhydride and H_2SO_4 (both A.R.). After 10 min. the two tubes are withdrawn and 2 ml. of the acetic anhydride-sulphuric acid mixture are pipetted into the extract. The contents of the tube are well mixed and the tube is placed in the water bath at 18° in the dark. After 60 min. the tube is removed and its percentage absorption of light of wave-length 640 mμ is measured in the Bolton-Williams type of photoelectric colorimeter, using a blank consisting of 5 ml. of chloroform and 2 ml. of acetic anhydride-sulphuric acid mixture. The quantity of cholesterol per 100 ml. of blood or serum can then be read off from calibration charts previously prepared, using standard quantities of cholesterol under the same conditions.

For photometric measurements, using the Pulfrich photometer, one cell is filled with the blank solution and the unknown is pipetted into the other. The percentage transmission (T) is read (see manufacturer's directions).

Observed $= (-\log (T/100))/L$, where L = length of cell.
$E_{sp.}$ = specific extinction coefficient of cholesterol.
Then $E/E_{sp.}$ = mg. cholesterol in sample.

The amount of cholesterol per 100 ml. blood = 500 times the amount in sample.

For the ordinary optical colorimeter, standards containing 0·5, 0·4 and 0·3 mg. cholesterol per 5 ml. are prepared and the colour is developed simultaneously with the test solutions. The test is compared with the standards and the results are obtained by the usual S/T ratios.

Preparation of silica for adsorption tube

The more obviously iron-containing particles are removed by hand from ordinary silica gel as purchased. The remaining particles are crushed with the aid of a mortar and pestle and are then transferred to a large flat-bottomed pyrex flask and more than covered with H_2SO_4 (1 vol. acid to 2 vol. water). The flask is next heated with constant shaking for about 20 min. in the fume cupboard. When cold the H_2SO_4 is decanted off and the silica washed several times with warm water; for the final washing the silica is left standing in contact with the water overnight. The purified silica is dried in a steam-heated oven for several hours. Only those particles of the pure silica which pass through a mesh $\frac{1}{20}$ in. diameter and yet not through one $\frac{1}{32}$ in. are used for the adsorption tube.

SUMMARY

1. It has been demonstrated that existing methods for the development of the green colour by the interaction of acetic anhydride and sulphuric acid with cholesterol in chloroform solution do not sufficiently take into account all the factors concerned. These factors have been studied, and from the results obtained an improved method of colour development has been suggested.

2. It has also been demonstrated that the yellow-brown colour in ether-alcohol extracts from biological material, which has been so frequently referred to, can be removed without loss of cholesterol.

3. On the basis of these facts an improved method for the colorimetric estimation of cholesterol in biological material has been described.

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30. SOME APPLICATIONS OF PERIODIC ACID TO THE STUDY OF THE HYDROXYAMINO-ACIDS OF PROTEIN HYDROLYSATES

1. THE LIBERATION OF ACETALDEHYDE AND HIGHER ALDEHYDES BY PERIODIC ACID

2. DETECTION AND ISOLATION OF FORMALDEHYDE LIBERATED BY PERIODIC ACID

3. AMMONIA SPLIT FROM HYDROXYAMINO-ACIDS BY PERIODIC ACID

4. THE HYDROXYAMINO-ACID FRACTION OF WOOL

5. 'HYDROXYLYSINE'

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Largely because of the lack of satisfactory analytical procedures, the hydroxyamino-acids are at present the least well investigated category of protein constituents, in spite of the fact that the simplest hydroxyamino-acid, serine, was one of the earlier amino-acids to be discovered in proteins [Cramer, 1865].

Nicolet & Shinn [1939] showed that periodic acid, a reagent introduced into organic chemistry by Malaprade [1934] for the splitting of α-glycols, also splits serine, the products of oxidation being one molecule of formaldehyde and, probably, one molecule each of ammonia and glyoxylic acid. Van Slyke *et al.* [1940] have subsequently used this reagent for the study of the new base 'hydroxylysine', and have mentioned a simple method for the detection of amino-acids having an —OH and an $—NH_2$ group in adjacent positions, by the liberation of NH_3 in the presence of periodate in strong K_2CO_3 solution.

Block & Bolling [1939] have made use of lead tetra-acetate, which reacts with α-glycols in the same way as periodic acid [Criegee *et al.* 1933], to determine threonine in protein hydrolysates. These workers determined the acetaldehyde liberated by a specific colorimetric method.

In the present work we have studied the reaction between periodic acid and hydroxyamino-acids both in solutions of known composition and in preparations derived from proteins.

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This has led to the development of analytical procedures which, though quantitatively not all that could be desired, are of high specificity, rapid, unlaborious and require only small amounts of material. They promise, therefore, to be of use in the study of proteins and preparations derived therefrom both for the analysis of complete hydrolysates and for the control of isolative procedures.

1. THE LIBERATION OF ACETALDEHYDE AND HIGHER ALDEHYDES BY PERIODIC ACID

[Cf. Martin & Synge, 1940]

On treatment with periodic acid, threonine yields acetaldehyde, and this may be separated from the formaldehyde resulting from serine, 'hydroxylysine' etc. by simple aeration, since formaldehyde, unlike its higher homologues, is not significantly volatile from aqueous solution [cf. Blair & Ledbury, 1925; Ledbury & Blair, 1925; Walker, 1940].

So far as we are aware, this difference between formaldehyde and its homologues has not previously been made use of for analytical purposes.

Experimental

In the present work, acetaldehyde was aerated from the reaction mixture in a 50 ml. washing tube directly into an absorption tower (separating funnel packed with glass beads and mounted in a Büchner flask, as shown in Fig. 1) containing 25 ml. of 0·3 % aqueous $NaHSO_3$. After rapid aeration for 30 min. the solution and washings from the tower were titrated in the Büchner flask with $N/300$ iodine solution [Friedemann & Kendall, 1929].

By placing a second absorption tower in series with the first, it was found that 95 % absorption of acetaldehyde took place in the first tower. The figures for volatile aldehyde given subsequently in this paper are not corrected for this and refer to aldehyde as titrated in the single absorption tower.

On aeration of 5·35 μmol. of acetaldehyde from 25 ml. of water in the aeration vessel, recoveries of 87–92 % were obtained, and these were not affected by the presence of 8·75 μmol. of formaldehyde in the acetaldehyde solution. Blank runs showed that the laboratory air was substantially free from aldehyde, and in no case was a 'reagent blank' found.

Yield of acetaldehyde from threonine

In all these tests synthetic *dl*-threonine was used. *dl-allo*-Threonine, prepared from *O*-methyl-*dl-allo*-threonine [cf. West & Carter, 1937], gave an identical yield, while samples of threonine of natural origin prepared from rabbit myosin by Sharp [1939] gave 70–72 % of the yield given by the synthetic material.

Amounts of 0·5–1·5 mg. of threonine were treated at room temperature with 50 mg. of periodic acid (B.D.H.) in 25 ml. total volume of aqueous solution. The yield of acetaldehyde obtained in the absorption tower after 30 min. rapid aeration was found to be dependent on *p*H, as shown in Table 1.

A definite optimum yield at about *p*H 7 is to be noted. The reasons for the lower yields on either side of this optimum, and for the incomplete yield at the optimum, have not been investigated in detail; in acid solution neither periodic acid, iodic acid nor ammonia interferes with the removal of added acetaldehyde

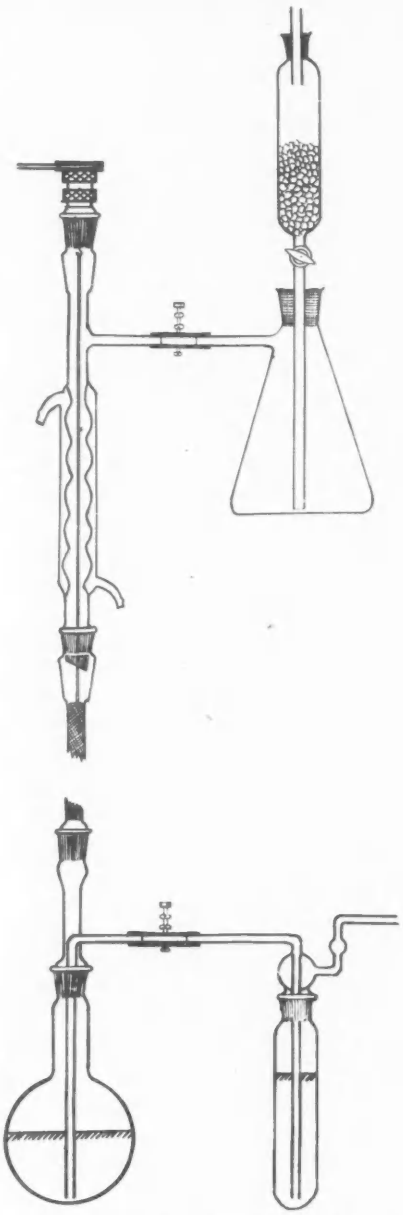

Fig. 1. Apparatus for micro-separation of aldehydes.

Table 1. *Yield of acetaldehyde from* dl*-threonine treated with periodic acid in various solutions*

Solutions used	Yield of acetaldehyde %
Water	9–10
M/5 acetate buffer, *p*H 4·7	52
M/5 $NaHCO_3$	66–72
M/5 phosphate buffer, *p*H 7·2	69
'Old' *M*/5 $NaHCO_3$, become more alkaline by loss of CO_2	55
50 % w/v K_2CO_3 solution	45

from aqueous solution. It is therefore probable that, in acid solution, threonine is either only slowly attacked by periodic acid or that side reactions result in products other than acetaldehyde. On the alkaline side it seems possible that the simultaneously liberated ammonia interferes with the determination, or that acetaldehyde is slowly oxidized by the reagents.

All determinations were accordingly carried out in fresh *M*/5 $NaHCO_3$ solution, which, on addition of periodic acid and the sample for analysis (itself usually acid), maintained the *p*H of the reaction mixture at about 7 at least during the earlier stages of the aeration.

The aldehyde material obtained from *dl*-threonine was identified qualitatively as acetaldehyde by conversion into the 2:4-dinitrophenylhydrazone.

Yield of volatile aldehyde from other amino-acids

dl-Serine, under the same conditions, yielded no aldehyde in the absorption tower, nor did *dl*-alanine, *l*-cystine, *l*-methionine, *l*-hydroxyproline, *i*-*β*-hydroxyglutamic acid, *l*-tyrosine, *l*-tryptophan, *l*-arginine, *dl*-lysine or *l*-histidine treated separately under the same conditions.

Yield of acetaldehyde from dl*-threonine in the presence of other amino-acids*

From a mixture of 0·78 mg. of *dl*-threonine and 2·9 mg. of *dl*-serine (Hofmann) 70 % of the aldehyde calculated for the threonine present was obtained.

In order to control more rigidly the specificity of the determination before applying it to protein hydrolysates, a mixture of amino-acids was made up, as shown in Table 2, to simulate a wool hydrolysate. This mixture was refluxed

Table 2. *Mixture of amino-acids used in control experiments*

Amino-acid	N (mg.)	N as % of total N
Ammonia (as NH_4Cl)	26·4	5·3
dl-Alanine	32·4	6·4
l-Arginine (as hydrochloride)	96·9	19·4
l-Aspartic acid	11·7	2·3
l-Cystine	59·4	11·8
l-Glutamic acid	44·6	8·9
Glycine	83·0	16·5
l-Histidine (as hydrochloride)	9·0	1·8
dl-Leucine	51·8	10·3
dl-Lysine (as dihydrochloride)	22·3	4·4
l-Methionine	6·6	1·3
dl-Phenylalanine	12·8	2·6
l-Tryptophan	5·3	1·1
l-Tyrosine	16·2	3·2
dl-Valine	23·4	4·7
Total	501·8	100

for 24 hr. in 6*N* HCl, and then evaporated to dryness *in vacuo*; the residue was dissolved in water, and again evaporated to dryness. An aliquot of this *material A*, containing 40 mg. N, was treated with 200 mg. of periodic acid in excess of $NaHCO_3$ solution, and yielded only 0·44 μmol. of volatile aldehyde, equivalent (1 mol. aldehyde = 1 atom N) to 0·014 % of the N in the reaction mixture.

When 10·1 mg. of *dl*-serine were added to the same mixture, no additional aldehyde was recovered. The addition of 9·5 mg. of *dl*-threonine gave volatile aldehyde equivalent to 70 % of the threonine added.

Volatile aldehyde from protein hydrolysates

In view of the satisfactory character of the above control experiments, it was decided to apply the determination to protein hydrolysates. The HCl hydrolysate of about 30 mg. of protein (after removal of excess HCl by repeated evaporation with water *in vacuo*) was treated with 50 mg. of periodic acid in 25 ml. *M*/5 $NaHCO_3$ solution in the usual manner. The volatile aldehyde obtained from a number of proteins is shown in Table 5 as N in percentage of the total N of the protein. In the cases of wool, casein, gelatin and wheat gluten, the recovery of additional aldehyde from threonine added to the reaction mixture with the protein hydrolysate was 65–70 %, substantially the same as in the absence of the hydrolysate. The above conditions were therefore adopted as standard for the determination.

Effect of concentration of reagents on yield of aldehyde

Increasing the concentration of periodic acid tenfold had no effect on the yield of volatile aldehyde, while similar increases of concentration of both protein hydrolysate and periodic acid (i.e. 300 mg. protein and 500 mg. periodic acid in 25 ml. *M*/5 $NaHCO_3$) in the case of some proteins, though not of others, lowered the percentage yield of aldehyde. The most extreme lowering observed (30 %) occurred in the case of wool.

Discussion

Although successful in differentiating threonine from other hydroxyamino-acids such as serine, β-hydroxyglutamic acid, 'hydroxylysine' etc. the method outlined above cannot be expected to differentiate threonine from its immediate higher homologues. For this reason, in view particularly of the *ad hoc* character of the authentication of threonine as the result of a research on the nutritive properties of amino-acids [McCoy *et al.* 1935], as well as the reported, though unconfirmed, isolations of higher hydroxyamino-acids [cf. Vickery & Schmidt, 1931; Dunn, 1938], it seemed to us desirable to investigate the qualitative composition of the volatile aldehyde material which we obtained from the various protein hydrolysates. Employing the conventional 2:4-dinitrophenylhydrazone identification (aeration into a saturated solution of 2:4-dinitrophenylhydrazine in 2*N* HCl, followed by recrystallization of the resulting precipitate from alcohol), we obtained, in the cases of wool, casein and gelatin, only the dinitrophenylhydrazone of acetaldehyde, which was identified by melting point, mixed melting point and X-ray powder photographs. In the case of wheat gluten, however, a mixture of the usual yellow crystals of acetaldehyde dinitrophenylhydrazone with redder crystals resembling those of the propaldehyde derivative was obtained. The X-ray powder photographs given by this mixture differed from those given by the three other preparations, and were closely similar, as pointed out by Dr F. O. Bell in an Appendix to this paper, to the

photograph given by a mixture of about 3 parts of acetaldehyde dinitrophenylhydrazone to 1 part of the corresponding propaldehyde derivative. This conclusion was confirmed by the melting-point data, and in the preliminary note of this work [Martin & Synge, 1940] it was therefore suggested that a higher homologue of threonine, β-hydroxy*nor*valine, might be present in wheat gluten.

It seemed desirable to examine the matter quantitatively, and for this purpose we developed the method outlined below for the separation of acetaldehyde and propaldehyde on the micro scale.

Micro-separation of acetaldehyde and propaldehyde

The problem of separating successive members of a homologous series on the micro scale by physical means has not to our knowledge previously been attempted. It seemed to us that a simple method was available if it was possible to find a solvent of intermediate boiling point from which the substances to be separated could be differentiated by chemical means and for which Raoult's vapour pressure law was approximately obeyed. If a mixture of the two solutes were then allowed to reflux in excess of the solvent in an efficient fractionating column, the more volatile substance should be concentrated at the top of the column, while the less volatile should remain in the distilling vessel and the lower part of the column.

We accordingly set up the apparatus illustrated in Fig. 1, which incorporates a Lesesne & Lochte [1938] spinning band column, for which high efficiencies have been demonstrated by Baker *et al.* [1940]. The apparatus was made of pyrex glass; the spinning band was of aluminium and was driven through a pulley mounted on a Quickfit & Quartz bearing and gland with standard brass-glass conical joint. The column was about $1\frac{1}{2}$ m. long, and was lagged with wool. The material for analysis was aerated into the solvent in the distilling vessel, which was cooled with solid CO_2, thus trapping the aldehydes. The distilling vessel was then placed in a water bath and the solvent was allowed to reflux with the band spinning at about 1000 r.p.m. while a current of air was drawn through the apparatus at a controlled rate of 120 bubbles per min. The air from the condenser passed to the absorption tower containing $NaHSO_3$, the aldehyde trapped being determined as already described.

As the boiling points of acetaldehyde and propaldehyde are 21° and 49° respectively, ether (B.P. 37°) seemed the most suitable solvent to employ. However, commercial ether contains appreciable quantities of aldehyde, and ether freed from aldehyde by the procedure of Carey *et al.* [1933] (washing with 30% $NaHSO_3$ followed by 10% NaOH) was found to contain aldehyde again after standing in the laboratory for a few days.

In view of this we tried to use light petroleum which had been fractionated in an efficient distilling column (B.P. 34°). This did not give rise to aldehyde. With petroleum in the column, however, both acetaldehyde and propaldehyde were recovered quantitatively in the absorber, as both appear to form low-boiling azeotropes with petroleum.

As Hildebrand [1921] has shown thermodynamically, deviation from Raoult's law is to be expected whenever two liquids on mixing show volume or heat changes. Liquids are likely to obey Raoult's law when the ratios $\frac{\text{temperature coefficient of expansion}}{\text{compressibility}}$ are similar for both pure liquids and their mixtures. This ratio is a measure of the polarity of the liquids. Lecat [1930; 1932] has made an extensive empirical study of the phenomenon of azeotropy in binary mixtures, and has summarized the literature, but we have not been

able to find a treatment of the phenomenon in terms of molecular physics. It is clear, however, that azeotropy is to be expected in mixtures of liquids of unequal polarity and is related to the formation of separate liquid phases. The use of azeotropic distillation in the manufacture of absolute alcohol is well known. Recently Matuszak & Frey [1937] have used distillation with SO_2 for the separation of butane isomers from butene isomers; Schicktanz *et al.* [1939] have used CCl_4 for the separation of the lower aliphatic alcohols in fusel oil, and [1940] benzene and toluene for the separation of the lower fatty acids. It is evident that the extreme form of azeotropic distillation is the distillation of two liquid phases, as in the steam distillation of oils, and that azeotropic distillation depends not only on the volatility but also on the polarity of the substances to be separated, and thus offers some of the advantages of liquid extraction.

Owing to the azeotropy of the system propaldehyde-petroleum we were compelled to use ether as solvent; when this had been extracted with $NaHSO_3$ followed by NaOH it gave satisfactory blanks for aldehyde on distillation, although it proved impossible to estimate the propaldehyde remaining in the ether in the apparatus by direct extraction of this with $NaHSO_3$ solution, since, with ether, an aldehyde titration was always obtained on shaking with the solution in a separating funnel, and this increased as the extraction was prolonged. We were therefore forced to estimate propaldehyde etc. remaining in the apparatus by the difference between the aldehyde estimated by direct aeration and by aeration through the column. We obtained positive evidence for its presence by adding petroleum to the ether in the apparatus after the acetaldehyde had been removed. In the time employed about two-thirds of the propaldehyde present was in this way collected at the top of the column.

The results of the control experiments are shown in Table 3.

Table 3. *Aldehyde micro-separation. Control experiments*

	Aldehyde taken in μmol.		Aldehyde found in μmol.	
Exp.	Acetaldehyde	Propaldehyde	A	B
I	0·0	0·0	0·0	0·5
II	12·9	28·0	14·8	—
III	26·4	0·0	26·6	0·5
IV	0·0	29·5	0·8	17·0
V	26·0	1·3	29·6	1·6

In these experiments the aldehydes were first collected in 100 ml. of aldehyde-free ether cooled with solid CO_2. The ether was then allowed to reflux in the column while a stream of air was drawn through the apparatus for 1 hr. at a rate of 120 bubbles per min. in the wash-bottle (Fig. 1), which contained water. At the end of this period the aldehyde collected in the absorber was titrated, giving the values shown in column A of Table 3. 100 ml. of aldehyde-free low-boiling petroleum were then added to the distilling vessel and aeration was continued for 2 hr. The aldehyde in the absorber was then again titrated, giving the values shown in column B. It will be seen from Exp. IV that about two-thirds of the propaldehyde present is carried up in the second stage of the distillation, and from comparing Exps. III and V that as little as 5% of propaldehyde in 1 mg. of acetaldehyde may readily be detected.

A control experiment with *iso*valeraldehyde showed that it did not reach the top of the column even after adding the petroleum; however, the autoxidation of aliphatic aldehydes higher than propaldehyde renders this analytical procedure unsuitable if a stream of air is employed.

The experiments with protein hydrolysates are shown in Table 4. Here the HCl hydrolysate of about 0·3 g. of protein was used, freed from excess of HCl by evaporation *in vacuo* and treated with 300 mg. of periodic acid in 10 ml. sat. aqueous $NaHCO_3$ solution. This mixture was aerated, and the volatile aldehyde was collected in cooled ether as already described. The experiments were then carried out exactly as the controls.

Table 4

Protein	Plant	Natural order	Vol. aldehyde as N in % of total N (cf. Table 5)	μmol. aldehyde found	
				A	B
Gluten	Wheat (*Triticum vulgare*)	Gramineae	1·2	26·2	0·7
Zein	Maize (*Zea Mays*)	Gramineae	1·2	26·9	0·9
Leaf protein	Cocksfoot (*Dactylis glomerata*)	Gramineae	2·2	47·6	0·8
Seed globulin	Peanut (*Arachis hypogaea*)	Leguminosae	1·1	36·0	1·0
Seed globulin (Edestin)	Hemp (*Cannabis sativa*)	Urticaceae	1·7	30·2	0·6
Seed globulin	Castor bean (*Ricinus communis*)	Euphorbiaceae	1·5	35·9	0·7

The results make it unlikely that in any case more than 3% of the aldehyde material was higher aldehyde than acetaldehyde. In each case rather more aldehyde was obtained as in column A than by direct aeration from the reaction mixture into the absorption vessel. There is thus no evidence for the occurrence in these proteins of higher homologues of threonine.

Discussion

Our earlier report of propaldehyde was based on melting-point and X-ray data on the dinitrophenylhydrazone material derived from wheat gluten. It subsequently came to our notice that acetaldehyde 2:4-dinitrophenylhydrazone is dimorphic [cf. Bryant, 1933]: this has been questioned by Campbell [1936]. The metastable form, obtained from the melt or by rapid evaporation from solution, bears a close resemblance to propaldehyde dinitrophenylhydrazone being decidedly more deeply coloured (red) than the stable form (yellow). In the light of this, we found that if authentic acetaldehyde and dinitrophenylhydrazine were allowed to react in HCl solution through which air from the reaction mixture with periodic acid of a wheat gluten hydrolysate had been drawn for sufficiently long to remove completely all acetaldehyde, propaldehyde etc. the resulting dinitrophenylhydrazone had a tendency to crystallize from alcohol in the metastable form even when crystallization was slow. It seems therefore that a trace of some slightly volatile contaminant promotes, in the case of the material from wheat gluten (not with casein, wool or gelatin), crystallization of the acetaldehyde derivative in the metastable form.

The dinitrophenylhydrazone material derived from wheat gluten, on chromatographic analysis in petroleum solution on MgO, gave two fractions very readily—a small proportion of red or brown material being very strongly adsorbed. The material obtained after the removal of this component showed, however, the same tendency to crystallize in the metastable form. The development of the chromatogram was not carried sufficiently far to permit of the separation of the acetaldehyde and propaldehyde derivatives, though control experiments suggested that this separation can be effected chromatographically; this requires long development, and the bands become too diffuse for the satisfactory visual detection of a small proportion of one component.

The various dinitrophenylhydrazone materials have been investigated by Dr F. O. Bell by the X-ray powder method; she presents her findings in an Appendix to this paper.

We conclude: (1) that the amount of propaldehyde obtained in this way from wheat gluten is at most small; (2) that acetaldehyde 2:4-dinitrophenylhydrazone, a compound much used by biochemists for the formal identification of acetaldehyde, has disadvantages for this purpose because of its dimorphism, which is now thoroughly substantiated by the X-ray work.

Criticism of the new procedure

The method for the determination of threonine, outlined above, is compared here with the method of Block & Bolling [1939].

(1) It requires the use of rather more material, though it remains on the micro scale.

(2) It is more rapid and less laborious. In particular it does not necessitate the transfer of material to an anhydrous solvent; the reaction is carried out in aqueous solution.

(3) The method has been shown to have a high specificity, threonine being the only known protein constituent to give volatile aldehyde under the conditions used for the determination. Block & Bolling report with their method interference by alanine and serine.

(4) It seems probable that higher homologues of threonine, if present, would be estimated by the procedure described here, though not by the procedure of Block & Bolling. To detect the presence of these we have developed the micro-separation technique described above, and the results suggest that higher homologues of threonine are absent from a number of proteins. We should like to emphasize the applicability of the type of separation described here for aldehydes to micro-separations in other homologous series. In its simplest form, a carrier of intermediate boiling point is required, from which the substances under analysis may be separated and determined by chemical means. It is necessary that at least one, and preferably both, of the substances to be separated should not show positive azeotropy with the solvent. (For separating substances of *different* chemical classes on the micro scale by distillation, 'carrier' solvents showing azeotropy with one class but not with the other may greatly facilitate separation, as has been shown by Matuszak & Frey [1937], and in this case it is not always necessary for the solvent to be of intermediate boiling point.)

(5) The yield of acetaldehyde obtained from threonine by our procedure seems to be about 70 % of the theoretical and to be largely independent of the presence of other amino-acids. It is impossible to deduce from Block & Bolling's paper what yield of acetaldehyde was obtained by them, since they used lithium lactate for checking the method; they found lithium lactate to give, under the same conditions as threonine, the same yield of acetaldehyde. Macara & Plimmer [1940] have recently reported experiments using the method of Block & Bolling. They found certain modifications of the procedure to be necessary, and failed to obtain very consistent results.

2. DETECTION AND ISOLATION OF FORMALDEHYDE LIBERATED BY PERIODIC ACID

We have not so far found a satisfactory method for determining the formaldehyde resulting from the action of periodic acid on serine etc.

Nicolet & Shinn [1939] obtained a good yield of formaldehyde through the dimedone derivative [Vorländer, 1929], after acting on serine with periodic acid, and, as shown below (Exp. I), we can confirm this result. The yield, however, is substantially reduced in the presence of a second amino-acid, such as tyrosine, which does not react with periodate, as shown in Exp. II, while in a complex mixture of amino-acids of known composition (Exp. III) no precipitation of formaldehyde-dimedone takes place. This can be ascribed to competition between the dimedone and the amino-acids for combination with the formaldehyde; in view of Exp. III it is not surprising that a wool hydrolysate, after treatment with periodate and removal of acetaldehyde by aeration, gave no precipitate on treatment with dimedone.

Nevertheless, though the dimedone method seems valueless for determining serine etc. in complete protein hydrolysates, it has proved of some value, as shown in Sections 4 and 5 of this paper, in setting lower limits to the purity of preparations derived from protein containing a high proportion of those amino-acids that yield formaldehyde on treatment with periodic acid.

Experimental

Exp. I. 10·8 mg. of *dl*-serine were treated with 50 mg. of periodic acid in 25 ml. of *M*/5 $NaHCO_3$ at room temperature. After a few minutes, 60 mg. of dimedone (B.D.H.) were added. When the dimedone had dissolved, the mixture was acidified with acetic acid, and after an hour the resulting precipitate was filtered off, washed and dried. It weighed 26·9 mg., corresponding to a yield of 90% of 1 mol. of formaldehyde per mol. of serine.

Exp. II. 10·1 mg. of *dl*-serine were treated exactly as in Exp. I except that 24·5 mg. of *l*-tyrosine were also present in the reaction mixture. The resulting precipitate weighed 9·4 mg., corresponding to a yield of 33% of 1 mol. of formaldehyde per mol. of serine.

Exp. III. A portion of *material A* (cf. Section 1) containing altogether 40 mg. N was added to 10·1 mg. of *dl*-serine and was treated with 200 mg. of periodic acid in the presence of excess of $NaHCO_3$ solution. (Volume of reaction mixture, 27 ml.) On adding dimedone and acetic acid as in Exps. I and II, no precipitate resulted.

3. AMMONIA SPLIT FROM HYDROXYAMINO-ACIDS BY PERIODIC ACID

Van Slyke *et al.* [1940] reported that 1 mol. of NH_3 is instantaneously set free from serine, threonine, β-hydroxyglutamic acid and 'hydroxylysine' by periodic acid in strong aqueous K_2CO_3 solution. They determined the NH_3 liberated by the aeration method of Van Slyke & Cullen [cf. Peters & Van Slyke, 1932]. Macara & Plimmer [1940] have recently employed this method for the determination of amino-acids of this type in hydrolysates of fractions derived from casein. They too report quantitative recovery of NH_3, although details of the experimental conditions are not given. Since the present work was carried out, we have received from Dr Van Slyke, in a private communication, details of his procedure; this involves the use in the reaction mixture of NaOH as well as K_2CO_3; a reagent is also added to prevent, by competition, condensation of NH_3 with the aldehydes also formed in the reaction. Nevertheless, while the experi-

ments described here are quantitatively imperfect, they serve to demonstrate the specificity of the method, and the results obtained with protein hydrolysates are at least of comparative significance.

Experimental

In these experiments we used simple aeration tubes (as shown in Fig. 1) without the perforated bubblers of Van Slyke & Cullen. Absorption of ammonia in the standard acid was found to be 92%; all the results obtained below have been corrected for this loss. This cruder apparatus seems to have an accuracy of about $\pm 5\%$, but in view of the low yields of NH_3 we were content with this.

Exp. I. 5·92 mg. of *dl*-serine in 10 ml. of 50% w/v aqueous K_2CO_3 solution with 50 mg. of periodic acid gave on aeration at room temperature 88% of 1 mol. of NH_3. Under the same conditions, equivalent quantities of the following amino-acids gave the yields of NH_3 stated: threonine, 83%; *dl-allo*threonine, 86%; *i*-β-hydroxyglutamic acid, 71%.

Exp. II. An amount of *material A* (cf. Section 1) containing 7·6 mg. N was added (as 0·5 ml. of aqueous solution) to 8 ml. of 62% w/v aqueous K_2CO_3. The ammonia set free was removed by aeration, and was found to be 102% of the amount calculated on the basis of the NH_4Cl used in making up this mixture.

100 mg. of periodic acid were then added, and the aeration was continued. No further ammonia was detected in the acid of the absorption vessel.

Exp. III. Exp. II was repeated, adding known quantities of hydroxyamino-acids to the mixture before carrying out the determination. On adding 5·92 mg. of *dl*-serine, 72% of 1 mol. of NH_3 was recovered on treatment with periodic acid; on adding 7·6 mg. of threonine, 77% of 1 mol. of N_3 was recovered.

Table 5. *Hydroxyamino-acid determinations on various protein hydrolysates*

Protein	N % of protein	As % of total N of protein: 'Amide-N'	NH_3-N lib. by periodate	'Threonine-N' (uncorrected)
Wool: Merino 64's I	16·7	8·2	9·2	3·3
Merino 64's II	—	—	—	3·5
Cotswold*	16·55	8·7	9·2	3·0
Commercial pure wool flannel	16·6	8·3	9·9	2·8
Silk fibroin	18·3	2·2	9·4	0·4
Myosin (68)†	16·8	7·5	4·3	1·9
Myosin (KB)†	16·8	7·9	4·5	2·0
Myosin (X)†	16·8	7·4	4·1	1·9
Myogen (C_2)†	16·8	7·0	5·4	2·4
Myogen (D)†	16·8	5·7	5·3	1·9
Gelatin (Coignet Gold Label)	—	—	3·4	1·0
Casein B.D.H.	13·4	10·4	6·3	2·1
Egg albumin	15·8	7·9	7·2	1·6
Wheat gluten B.D.H.	13·5	23·3	5·3	1·2
Zein	16·3	19·7	5·9	1·2
Leaf protein: *Dactylis glomerata*	13·1‡	7·5	5·4	2·1
Seed globulin: *Arachis hypogaea*	—	12·3	5·0	1·1
Cannabis sativa	18·55	11·0	5·4	1·7
Ricinus communis	—	11·1	5·3	1·5

* Before analysis cystine had been removed from the hydrolysate by Cu_2O.

† These preparations of rabbit muscle proteins were provided by Dr J. G. Sharp of the Low Temperature Research Station, Cambridge. Myosin KB is a sample of myosin reprecipitated six times.

‡ On ash-free basis: the preparation contained 2·4% of ash.

On the basis of these experiments, we have adopted the following routine method for the estimation of the hydroxyamino-acid content of complete protein hydrolysates:

An amount of an HCl hydrolysate (from which excess of HCl has been removed by repeated evaporation with water *in vacuo*), corresponding to 60–70 mg. of protein and not exceeding 2 ml. in volume, is placed in the aeration tube with 8 ml. 62% w/v aqueous K_2CO_3 solution, and the 'amide-N' is determined by aeration into standard acid. 100 mg. of periodic acid are then added, and the aeration is repeated. The ammonia thus set free is taken as a measure of the hydroxyamino-acids of this type present. In most cases rapid aeration was not interfered with by frothing of the reaction mixture.

In Table 5 are set out parallel determinations of 'ammonia-N', 'N liberated by periodate' and 'threonine-N' as percentages of the total N of the proteins, as determined by the Kjeldahl procedure.

Discussion

All the indications are that the 'threonine' values set out in Table 5 are 30–35% low (cf. Section 1) and the 'N liberated by periodate' 20–30% low. The results are, at least, of comparative significance, and set lower limits to the amount of the amino-acids in question that are present.

In the cases of wool, casein, gelatin, gluten, cocksfoot leaf protein and the seed globulins of *Arachis*, *Cannabis* and *Ricinus*, there is good evidence that the volatile aldehyde set free is very largely acetaldehyde, and therefore probably derived from threonine (cf. Section 1).

The high content of hydroxyamino-acids in wool and silk fibroin, compared with the other proteins studied, is to be noted. In the case of wool, about one-third of this quantity appears to be threonine: silk fibroin, however, appears to have the lowest threonine content of all the proteins investigated. In each case, the difference between these values is probably mostly due to serine—in which silk fibroin is known to be comparatively rich [Fischer & Skita, 1902; Abderhalden, 1922]. In the present work (Section 4) it is shown that wool also contains considerable quantities of serine.

Decomposition of a proportion of this serine may be expected to account for part of the ammonia found in the acid hydrolysates of both these proteins [cf. Damodaran & Ramachandran, 1941].

From Table 5 zein would be expected to be fairly rich in serine. This is in agreement with the isolation from zein by Osborne & Jones [1910] of 1% of serine.

4. THE HYDROXYAMINO-ACID FRACTION OF WOOL

In this section we report the results of an investigation, using the analytical procedures developed above, of the fraction obtained from a wool hydrolysate by the acetylation-benzoylation procedure of Synge [1939]. In that paper, a fraction from wool was reported containing 5·3% of the N of the wool (96% of it amino-N). In a subsequent paper [Martin & Synge, 1941] we described a similar preparation (made after removal of the higher monoamino-acids from the hydrolysate by acetylation and chloroform extraction) containing 5·9% of the N of the wool (95% of it amino-N). This latter material, obtained as *N*-acetyl derivatives in the form of a nearly colourless glass, was employed in the present investigation.

Experimental

Some of the *N*-acetylated material was hydrolysed by refluxing for 3 hr. in 15 ml. of 2*N* H_2SO_4. This contained 122·5 mg. N, and all values given below refer to this original quantity, being corrected for aliquots removed for analysis.

$SO_4^=$ was then exactly removed with baryta, and the filtrate and washings from $BaSO_4$ were made up to standard volume. A threonine determination (cf. Section 1) on an aliquot of this material gave the threonine-N (uncorrected) as 9·2 % of the N of the fraction, i.e. 0·54 % of the total N of the wool.

The rest of the solution was concentrated by evaporation *in vacuo* and deposited crops of insoluble crystalline material, which were removed by filtration (tyrosine). This material amounted in all to 323 mg., equivalent to 25·0 mg. N, i.e. 20 % of the N of the fraction and 1·2 % of the N of the original wool.

The aqueous filtrate from tyrosine on addition of alcohol gave a syrup which slowly crystallized. These crystals were recrystallized from water-alcohol several times; after two crystallizations the threonine content (uncorrected) of the crystalline material had fallen to about 1 %, at which value it remained on further recrystallization.

The final product (*material S*) weighed 330 mg.

Analytical data on material S

Elementary analysis. Found: C, 31·8; H, 6·35; N, 12·4; ash, 6·6 %. The product therefore contained 40·8 mg. N, i.e. 33 % of the N of the hydroxyamino-acid fraction and 1·95 % of the total N of the wool. 'Corrected' for ash content, the analytical figures become: C, 34·1; H, 6·81; N, 13·3 % (calc. for serine, $C_3H_7O_3N$: C, 34·3; H, 6·66; N, 13·3 %).

Optical rotation. Material S had $[\alpha]_D^{17·5°} - 8·06°$ (water, $c = 5$). Fischer & Jacobs [1906] record *l*-serine as having $[\alpha]_D^{20°} - 6·83°$ (water, $c = 10$).

Formaldehyde on treatment with periodate. On treating 13·5 mg. of S with periodate and dimedone, as described in Section 2, 25·4 mg. of formaldehyde dimedone were obtained. On recrystallization this material had M.P. 188–189°, not depressed on admixture of authentic formaldehyde dimedone, for which Vorländer [1929] records M.P. 189°. (Found: C, 70·5; H, 8·33 %. Calc. for $C_{17}H_{24}O_4$: C, 69·9; H, 8·23.)

The yield of formaldehyde was thus equivalent to 73 % of the N of the material.

Ammonia on treatment with periodate. 7·2 mg. of *material S* gave, on treatment with K_2CO_3-periodate as in Section 3, 88 % of its N as NH_3—the same yield as that obtained from pure serine under the same conditions.

Material S gave a negative orcin-H_2SO_4 test for carbohydrate.

Discussion

From the above analytical data we conclude that *material S* consists largely of serine, contaminated by inorganic material. The fact that its optical rotation is significantly higher than that reported for *l*-serine by Fischer & Jacobs may be ascribed either to a contaminant of high laevorotation or to a raising of the rotation produced by the inorganic material present. It seems probable, nevertheless, that the acetylation-benzoylation procedure affords a method for isolating serine from proteins in the optically active state. This has not often been reported since Fischer [1907] obtained his active product from silk [cf. Posternak & Posternak, 1927; Lipmann & Levene, 1932].

The results of the present work on the hydroxyamino-acid fraction of wool are summarized in Table 6.

Table 6. *Composition of the hydroxyamino-acid fraction prepared from wool*

	N as % of N of fraction	N as % of N of wool
Identified as: serine	33	1·95
threonine (uncorrected)	9·2	0·54
tyrosine	20	1·2
Unidentified	37·8	2·2
Total	100	5·9

It seems likely that a large proportion of the unidentified N is serine, as unavoidable losses were encountered in working up *material S*.

By making use of the figures given in Table 5 for the original wool hydrolysate (Merino 64's I) it is possible to form an estimate of the yields obtained by the acetylation-benzoylation procedure.

The threonine content of the original hydrolysate (corrected—assuming the figure in Table 5 to be low by 30%) is 4·7% of total N. The threonine present in the hydroxyamino-acid fraction (from Table 6, corrected in the same way) is only 0·77% of the total N of the wool. This represents a recovery, in the hydroxyamino-acid fraction, of 16% of the threonine present in the hydrolysate.

The recovery of serine may be estimated as follows: assuming that the 'N liberated by periodate' as given in Table 3 is 25% low (cf. Section 3), we have 'N liberated by periodate' as 12·2% of the total N of wool. Subtracting the 'corrected' value for threonine-N of 4·7%, this leaves a maximum value for serine-N in the original hydrolysate of 7·5% of the total N. In the hydroxyamino-acid fraction, the upper limit for serine is (total N − threonine N (corrected) − tyrosine N) = 4·0% of total N of wool (cf. Table 6). On this basis, a recovery of about 50% of the serine of the original hydrolysate is obtained in the hydroxyamino-acid fraction.

This difference between the behaviours of serine and threonine may perhaps be ascribed to a more ready benzoylation of the primary alcoholic group of serine than of the secondary alcoholic group of threonine, in the course of preparing the hydroxyamino-acid fraction.

Nevertheless, in hydroxyproline, as shown by Synge [1939], the secondary alcoholic group is readily benzoylated, as is the phenolic —OH group of tyrosine.

5. 'HYDROXYLYSINE'

Van Slyke & Hiller [1921] reported the occurrence of a base of unknown constitution in the lysine fraction of a gelatin hydrolysate. Schryver *et al.* [1925] reported the isolation of a new base from isinglass, of which they prepared a tribenzoyl derivative. They suggested that it was a hydroxy derivative of lysine, and tentatively assigned the —OH group to the β-position, by analogy with serine and β-hydroxyglutamic acid. Van Slyke *et al.* [1938] reported the isolation of a base from the lysine fraction of a gelatin hydrolysate by fractional crystallization of the picrates; it had the composition of hydroxylysine and its picrate had M.P. 225°. More recently Van Slyke *et al.* [1940] have analysed the electrometric titration curve of the new base, and have shown that the compound is

split by periodic acid to give one molecule of formaldehyde and one molecule of ammonia. They suggest that the new base is either δ-hydroxy-αε-diaminohexoic acid or ε-hydroxy-αδ-diaminohexoic acid.

Shortly after the 1938 note of Van Slyke *et al.* one of us (R. L. M. S.) applied the acetylation-benzoylation procedure of Synge [1939] to the lysine fractions of gelatin and isinglass hydrolysates and in each case isolated a picrate corresponding to the description given by Van Slyke *et al.* [1938].

Experimental

Approximately 150 g. of gelatin (Coignet Gold Label) containing 21·2 g. N were hydrolysed by refluxing with 1·5 l. of 6*N* H_2SO_4 for 6 hr. The hydrolysate was cooled and treated with 300 g. of phosphotungstic acid (B.D.H.) at a final volume of 5 l. After keeping overnight in the ice-chest, the precipitate was filtered off and decomposed with baryta as usual. The filtrate and washings from the decomposition were concentrated *in vacuo* to remove NH_3, acidified to thymol blue with H_2SO_4 and treated with Ag_2SO_4 at 100° until excess had been added (brown precipitate on testing a portion with baryta). The solution was cooled, saturated with $Ba(OH)_2$ and filtered, and the filtrate, after acidification to thymol blue with H_2SO_4 (to remove Ba), was treated with H_2S to remove Ag. The filtrate from Ag_2S and $BaSO_4$ was freed from H_2S by concentration *in vacuo* and was then treated with 20 ml. of H_2SO_4 and 100 g. of phosphotungstic acid at a volume of 1·5 l. After keeping overnight in the ice-chest, the precipitate was filtered off and decomposed with baryta as usual. The filtrate was acidified with H_2SO_4 to precipitate Ba, and the filtrate from $BaSO_4$, the 'lysine fraction', was found to contain 4·3 % of the original N of the gelatin in the form of amino-N (½ hr. Van Slyke determination). This solution was then treated with two-thirds of the theoretical quantity of picric acid, and the resulting lysine picrate, after recrystallization from water, corresponded to 1·93 % of the total N of the original gelatin. The combined mother liquors and washings from this were freed from picric acid by acidification with H_2SO_4 and repeated extraction with chloroform. The aqueous residue was then brought to *p*H 8·5 with NaOH, concentrated to small volume *in vacuo* and subjected to the acetylation-benzoylation procedure [Synge, 1939], employing for the acetylation 200 ml. 2*N* NaOH and 20 ml. of acetic anhydride and for the benzoylation 185 ml. 2*N* NaOH and 23 ml. of benzoyl chloride. The resulting *N*-acetylated material was hydrolysed in 108 ml. 2*N* H_2SO_4 by refluxing for 3 hr., after which the solution was found to contain 57·5 mg. amino-N (½ hr. Van Slyke determination) equivalent to 0·27 % of the total N of the gelatin. 20 g. of phosphotungstic acid were added in 60 ml. of water. Precipitation of the resulting phosphotungstate, a fluffy, crystalline material (which did not readily settle, but filled the body of the solution), was slow, but had ended after keeping overnight in the ice-chest. The precipitate was filtered off and decomposed in the usual manner with baryta; the amino-N obtained was found (½ hr. Van Slyke determination) to correspond to 0·13 % of the total N of the gelatin.

This material yielded with picric acid 121 mg. of a crystalline picrate, which on recrystallization from water had explosion point 226–227° and amino-N (½ hr. Van Slyke determination) 7·38 %. ($C_6H_{14}O_3N_2.C_6H_3O_7N_3$ requires amino-N 7·17 %.)

A picrate having the same properties was obtained in the same way from isinglass (Boots 'Besyet'); the product resulting from decomposition of the final phosphotungstic acid precipitate contained amino-N equivalent to 0·15 % of the original N of the isinglass.

Several preparations obtained in this way were combined, and after the appearance of the 1940 note of Van Slyke *et al.* it seemed to us desirable to test the material for formaldehyde and ammonia on treatment with periodate.

A control experiment with synthetic *dl*-lysine [cf. Eck & Marvel, 1939] showed that on treatment with periodate lysine produces neither of these substances.

Formaldehyde liberated by periodate from the base. 57·3 mg. of the combined picrate preparations were freed from picric acid by extraction with chloroform from 10 ml. of 0·15*N* HCl. The aqueous residue was freed from chloroform by aeration, and treated with 1 g. of $NaHCO_3$ and 120 mg. of periodic acid at a volume of 30 ml.; 120 mg. of dimedone were then added, and when it was dissolved the mixture was acidified with acetic acid. After keeping 1·5 hr. the resulting precipitate was filtered off, washed and dried. It weighed 28 mg., corresponding to a yield of 65 % of 1 mol. of formaldehyde per mol. of 'hydroxylysine'. On recrystallization from alcohol the material had M.P. 187–188°, which was not depressed on admixture of authentic formaldehyde-dimedone. (Found: C, 69·6; H, 8·14 %. Calc. for $C_{17}H_{24}O_4$: C, 69·9; H, 8·23 %.)

Ammonia liberated by periodate from the base. After removal of picric acid as above, followed by removal of chloroform by aeration, an amount of the base corresponding to 16·8 mg. of picrate gave on treatment with K_2CO_3 and periodic acid as in Section 3 0·19 mg. of NH_3-N. This is equivalent to the liberation of 31 % of a mol. of NH_3 per mol. of 'hydroxylysine'.

Discussion

It will be seen that the above experiments confirm the discovery of Van Slyke and co-workers although the yields of formaldehyde and ammonia fall short both of the 'theoretical' and of those obtained with serine and threonine under the same conditions. The method employed for the isolation of the base is in itself an indication that the base possesses a free hydroxyl group.

It seemed of interest to obtain an estimate of the amount of the base possibly present in gelatin. A sample of the 'lysine fraction' obtained as described above from Coignet Gold Label gelatin was treated with K_2CO_3 and periodic acid as in Section 3. 0·20 % of the total N of the gelatin was obtained as ammonia, suggesting that at least 0·4 % of the total N of the gelatin is in the form of 'hydroxylysine'. In view of the low yield of NH_3 obtained in this way from our purest preparation of the base, the amount actually present may be considerably greater.

It appears therefore that the yield of 'hydroxylysine' isolated by the acetylation-benzoylation procedure from the 'lysine fraction' is not high (< 33 %), in spite of the enormous excesses of reagents which it was possible to employ. This, by analogy with the behaviour of serine and threonine (cf. Section 4) is perhaps an indication in favour of the alcoholic group being secondary, i.e. favours the view that 'hydroxylysine' is δ-hydroxy-αε-diaminohexoic acid rather than ε-hydroxy-αδ-diaminohexoic acid.

A parallel experiment on the 'lysine fraction' of wool (Merino 64's I) prepared in exactly the same manner gave < 0·01 % of the total N of the wool in the form of NH_3 liberated by K_2CO_3 periodate.

In a private communication Dr Van Slyke informs us that the hydroxylysine-N in gelatin and wool amounts respectively to 0·9 and 0·11 % of the total N (minimal figures based on N liberated by periodate from recrystallized phosphotungstic acid precipitates).

The formula suggested for hydroxylysine by Van Slyke and co-workers may be said to have a biological justification. The idea that proline may have the same biological origin as the basic amino-acids was held by its discoverer [Fischer, 1903]. Wada's [1933] demonstration of the conversion of citrulline into proline on boiling with acid lends support to this hypothesis (cf. also Sørensen's [1905] conversion of δ-hydroxy-α-aminovaleric acid into proline). It is not likely that this reaction occurs to any great extent during the acid hydrolysis of proteins, although since the work of Wada [1933] and Fearon [1939] citrulline is to be regarded as a probable constituent of proteins, and its breakdown during acid hydrolysis would account for the small amounts of CO_2 that are set free [cf. Dunn, 1925] as well as for some NH_3. The balance of evidence suggests that proline residues exist pre-formed in proteins. If, however, their biological precursor is to be regarded as an ornithine derivative, then the precursor of hydroxyproline would be a derivative of γ-hydroxyornithine, which is homologous with δ-hydroxylysine. On this view it is to be expected that the asymmetry of the γ-C atom of hydroxyproline is the same as that of the δ-C atom of hydroxylysine. Neither configuration has yet been ascertained. In this connexion it is of interest that Felix & Mager [1937] report the occurrence of small amounts of hydroxyproline in clupein, together with still smaller amounts of a base having the elementary composition of hydroxyarginine. In the present work it is shown that hydroxyproline and hydroxylysine, both present in a gelatin hydrolysate, can only be present in small quantity in wool.

Summary

1. Threonine on treatment with periodate at *p*H 7 yields 70% of 1 mol. of acetaldehyde. Acetaldehyde and higher homologues, unlike formaldehyde, may be removed from aqueous solution by simple aeration; this provides the basis of a micro-method for the determination of threonine and higher homologues in complete protein hydrolysates and other material of biological interest. The determination is not interfered with by serine, 'hydroxylysine' or other known naturally occurring amino-acids.

2. The aldehyde material obtained in this way from a number of proteins has been shown to consist almost entirely of acetaldehyde. We have therefore no evidence for the occurrence of higher homologues of threonine in these proteins.

3. To demonstrate this, we have developed a micro-method for the separation of acetaldehyde from its higher homologues, which depends on 'carrier' distillation using ether as a solvent. By this means as little as 5% of propaldehyde can be detected in 1 mg. of acetaldehyde. The principles of this type of separation, and its applicability to micro-separations in other homologous series, are discussed.

4. The explanation of our earlier report [Martin & Synge, 1940] of propaldehyde in the aldehyde material from wheat gluten appears to lie in the dimorphism of acetaldehyde dinitrophenylhydrazone. In an appendix, Dr Bell discusses the relevant X-ray data.

5. The conditions for precipitation by dimedone of the formaldehyde set free from serine etc. by periodic acid have been studied. The presence of other amino-acids seriously lowers the yield obtained.

6. The production of NH_3 by periodate from such hydroxyamino-acids as serine, threonine, β-hydroxyglutamic acid and hydroxylysine in the presence of K_2CO_3 [cf. Van Slyke *et al.* 1940] has been shown by control experiments to be

applicable to the estimation of the aggregate of these substances in complete protein hydrolysates.

7. The results of threonine and aggregate hydroxyamino-acid estimations on a number of proteins are tabulated and discussed.

8. Using the methods here developed, the hydroxyamino-acid fraction obtained from a wool hydrolysate by the acetylation-benzoylation procedure of Synge [1939] has been investigated. This procedure appears to give low recoveries of threonine. Nearly 2% of the N of wool was isolated in the form of fairly pure serine showing optical activity.

9. A preparation agreeing in properties with the 'hydroxylysine' of Van Slyke *et al.* has been obtained from the lysine fractions of gelatin and isinglass hydrolysates by the acetylation-benzoylation procedure. This material, like Van Slyke's, yields ammonia and formaldehyde on treatment with periodic acid.

We wish to thank Dr Bell and her colleagues for the X-ray investigations, and for the interest which they have taken in this work. We are also grateful to Dr K. Bailey for a de-cystinized hydrolysate of Cotswold wool, to Dr H. E. Carter for a sample of *O*-methyl-*dl*-*allo*threonine, to Prof. A. C. Chibnall for synthetic threonine and specimens of various proteins, to Prof. C. R. Harington for synthetic β-hydroxyglutamic acid, to Dr J. G. Sharp for specimens of natural threonine and of various muscle proteins, to Miss Thornton and Mr Warburton for preparing the figure, and to Miss M. I. Whitehead for help in preparing the paper for press.

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APPENDIX

X-RAY INVESTIGATION OF THE 2:4-DINITROPHENYLHYDRAZINE COMPOUNDS WITH VARIOUS ALDEHYDES AND KETONES AND WITH PRODUCTS LIBERATED BY PERIODIC ACID FROM PROTEIN HYDROLYSATES

By FLORENCE O. BELL

The 2:4-dinitrophenylhydrazone of the compound obtained from wheat gluten by the action of periodic acid (see above) is a mixture of yellow and orange-red crystals; acetaldehyde 2:4-dinitrophenylhydrazone as usually prepared by crystallization from solvents is yellow, and the propaldehyde derivative (however prepared) is red. The melting point of the gluten derivative is lower than that of the yellow acetaldehyde derivative, and it is therefore possible that the gluten compound is formed from acetaldehyde (derived from threonine) with small amounts of propaldehyde (derived from a higher homologue of threonine). In an attempt to determine the nature of the gluten compound, X-ray powder photographs were taken of the gluten 2:4-dinitrophenylhydrazine derivative, of the hydrazones formed with acetaldehyde, propaldehyde and acetone respectively, and of various mixtures of these derivatives.

Direct comparison photographs of specimens were taken on two halves of one and the same photographic film, the specimens being exactly interchangeable so that specimen-to-film distance, development conditions etc. were the same for both photographs.

Crystals of the gluten aldehyde derivative ground to a powder were compared with the powdered derivatives of acetaldehyde, propaldehyde and acetone

respectively, but the numbers, distribution and intensities of the lines in the gluten photograph were not the same as those of the other three compounds, although certain strong lines appeared in approximately the same positions in all four photographs: the gluten derivative was certainly not identical with the yellow crystals of the acetaldehyde derivative, whereas aldehydes derived similarly from casein, gelatin, wool and gliadin were in all cases identified, in the form of the 2:4-dinitrophenylhydrazone, as acetaldehyde. On the assumption, then, that in addition to acetaldehyde another aldehyde or ketone was present in the gluten preparation, the latter was compared with various mixtures of aldehyde and ketone derivatives.

It was noticed at this stage, however, that fused yellow crystals of acetaldehyde 2:4-dinitrophenylhydrazone give on cooling *red* crystals, similar to those of the gluten and propaldehyde derivatives, and that there are pronounced differences between the X-ray photographs (see Pl. 2, figs. 1, 2) of these yellow and red dimorphs. Further, we found that this dimorphism of acetaldehyde 2:4-dinitrophenylhydrazone had already been noted and studied crystallographically and optically by Bryant [1933].

In spite of this promising clue, the X-ray photograph of the gluten compound (also fused in this case) still did not correspond exactly with that of the red metastable acetaldehyde derivative (see Pl. 2, fig. 3), although it had similar intensities and distribution of lines. Small quantities of propaldehyde or acetone added to the acetaldehyde produced marked effects on the X-ray photograph of the 2:4-dinitrophenylhydrazone (see Pl. 2, figs. 4, 5), as would be expected from Bryant's results: he observed that very small amounts of propaldehyde mixed with acetaldehyde in the 2:4-dinitrophenylhydrazone modified the optical constants of the crystals considerably.

It seems clear, therefore, from these investigations that the aldehyde liberated from gluten hydrolysates by means of periodic acid is at any rate not pure acetaldehyde, since the photograph of its 2:4-dinitrophenylhydrazone does not agree strictly either with that of the yellow or with that of the red form of acetaldehyde 2:4-dinitrophenylhydrazone. No artificial mixture of aldehydes or ketones has so far been found which exactly corresponds to the gluten compound. A mixture of three parts of the acetaldehyde derivative and one part of the propaldehyde derivative crystallized out together from alcohol (see Pl. 2, fig. 6), and also a fused mixture of the acetaldehyde derivative with 2 % of the propaldehyde derivative, both gave X-ray photographs closely corresponding to that of the gluten compound, crystallized and fused respectively, but again neither was really identical with it. The presence of small quantities of acetone in the gluten compound appears definitely unlikely from the X-ray photographs. There is some contaminant, nevertheless, and moreover a contaminant that predisposes the acetaldehyde derivative to crystallize in the red form just as though it had been prepared from the melt; but what this contaminant is it must be confessed is still an open question. It is tempting to assume that a small proportion of propaldehyde or of some higher aldehyde is present, but the problem is so complicated by dimorphism and mixed crystallization effects that it would require much more time to investigate satisfactorily than is immediately available, so it is better to suspend judgment for the moment.

I am much indebted to Dr K. M. Rudall for taking a number of the X-ray photographs, and to Dr W. T. Astbury for the benefit of valuable discussion.

EXPLANATION OF PLATE 2

Fig. 1. Stable yellow acetaldehyde 2:4-dinitrophenylhydrazone.

Fig. 2. Comparison of metastable red acetaldehyde 2:4-dinitrophenylhydrazone (left) and propaldehyde 2:4-dinitrophenylhydrazone (right).

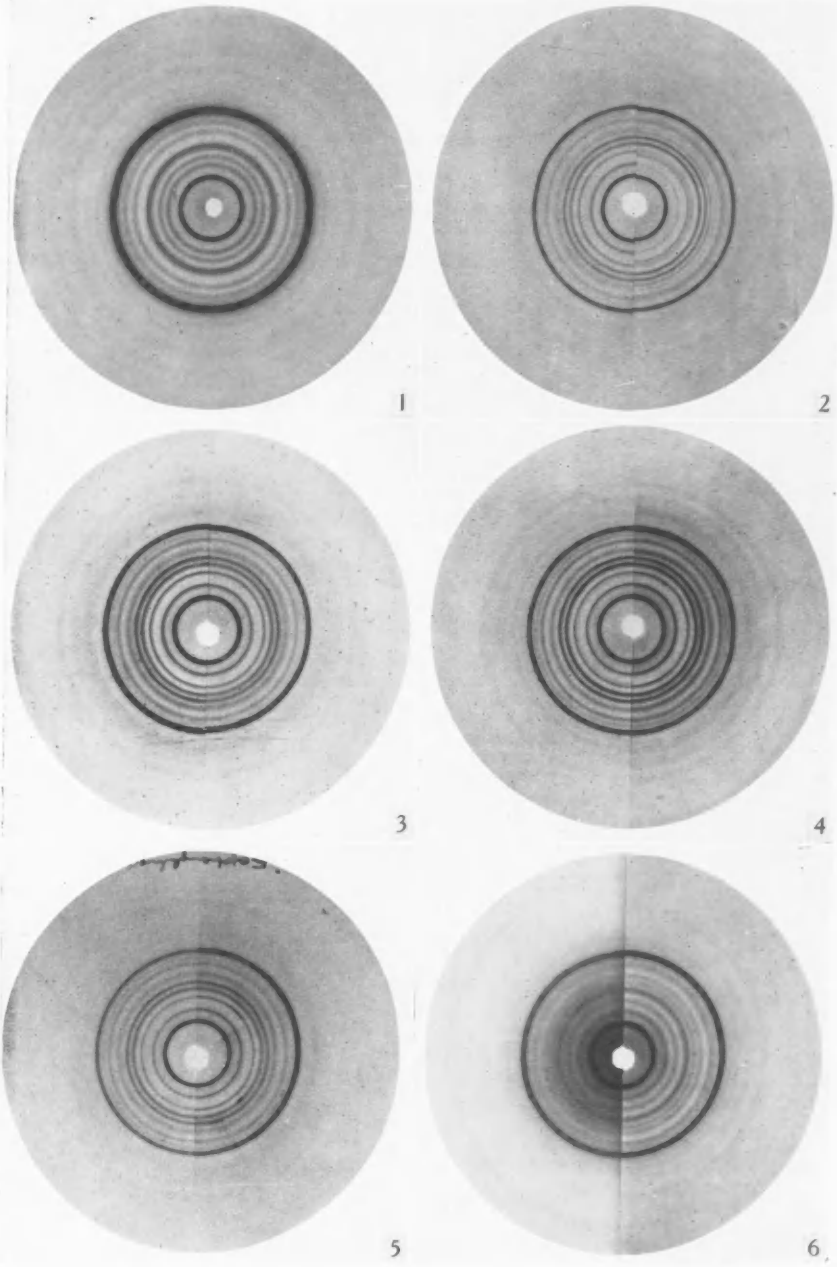
Fig. 3. Comparison of red acetaldehyde 2:4-dinitrophenylhydrazone (left) and fused gluten compound 2:4-dinitrophenylhydrazone (right).

Fig. 4. Comparison of red acetaldehyde 2:4-dinitrophenylhydrazone (left) and acetaldehyde 2:4-dinitrophenylhydrazone plus 2% propaldehyde 2:4-dinitrophenylhydrazone fused (right).

Fig. 5. Comparison of red acetaldehyde 2:4-dinitrophenylhydrazone (left) and acetaldehyde 2:4-dinitrophenylhydrazone plus 2% acetone 2:4-dinitrophenylhydrazone fused (right).

Fig. 6. Comparison of gluten compound 2:4-dinitrophenylhydrazone (left) and acetaldehyde 2:4-dinitrophenylhydrazone plus 25% propaldehyde 2:4-dinitrophenylhydrazone crystals (right).

All X-ray photographs taken with Cu K_α radiation, and specimen to film distance approximately 3 cm.



Figs. 1–6.

31. ANALYSIS OF PROTEINS

13. CASEO-PHOSPHOPEPTONE

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Posternak [1927, 1, 2] first prepared a polypeptide from casein with a P : N ratio of 4 : 15 or 16, and isolated from it the equivalents of 3 mol. glutamic acid, 0·6 mol. aspartic acid, 3 mol. *iso*leucine, 3 mol. serine and 4 mol. H_3PO_4. Evidently, on account of decomposition of serine during hydrolysis, he concluded that the phosphopeptone contained 4 mol. phosphoserine. Rimington [1927, 1, 2] isolated a nonapeptide which was considered to consist of 3 mol. hydroxyglutamic acid, 4 mol. hydroxyaminobutyric acid, 2 mol. serine and 3 mol. H_3PO_4. The composition of the phosphopeptone was further examined by Levene & Hill [1933], who obtained from it a dipeptide of glutamic acid and phosphoserine. Previously, Lipmann & Levene [1932] had isolated the Ba salt of phosphoserine from a polypeptide from vitellin and Lipmann [1933, 1, 2, 3] isolated it from a phosphopeptone from casein. Glutamic acid and serine were again isolated by Schmidt [1934]. The presence of glutamic acid in a phosphopeptone from vitellin could not be established by Levene & Schormüller [1933]. Sorimati [1939] considered that he obtained the Ba salt of phosphoserylglutamic acid. The only definite products from caseo-phosphopeptone thus seem to be glutamic acid and serine. On account of the contradictory results of Posternak and Rimington and in connexion with our work on dephosphocasein and dephosphocaseose [Macara & Plimmer, 1940] experiments were carried out on caseo-phosphopeptone. A preliminary account of our results was communicated to the Biochemical Society in March 1940. Caseo-phosphopeptone appeared to be an octapeptide with 2 mol. serine, 2 mol. glutamic acid and 2 mol. H_3PO_4. Rimington [1941] consequently re-examined his caseopeptone; he has consulted with us and kindly shown us his paper before publication. His product is now considered to be a nona- or deca-peptide with 5 mol. glutamic acid, 1 mol. *iso*leucine, 4 mol. serine and 3 mol. H_3PO_4. Owing to unforeseen circumstances our work had to cease, but we feel that our data, so different from those of Rimington, should appear simultaneously. Damodaran & Ramachandran [1941] have given the composition of caseopeptone as a decapeptide with 3 mol. glutamic acid, 4 mol. serine, 3 mol. *iso*leucine and 3 mol. H_3PO_4. There is thus some general agreement about the composition of the phosphopeptone, but the number of mol. of each amino-acid needs further examination.

Experimental

Preparation of caseo-phosphopeptone

Crude Na-phosphopeptone was prepared from 'Rhenania' casein, purchased by one of us many years ago, according to the method of Levene & Hill [1933], using portions of 50 g. casein and 1·5 g. trypsin (B.D.H.). The casein contained

10·1 % H_2O, 13·44 % N and 0·82 % P. The average yield was 3–4 g. or 6–8 %, with a N : P ratio of 4·5–5 : 1. Na_2CO_3 was invariably present.

For purification, several crops were combined, dissolved in water, the Na_2CO_3 neutralized with acetic acid, the Pb salt again precipitated and converted back into the Na salt. This was carried out three times and the Na salt thrown down with alcohol. The alcohol decanted from the syrupy precipitate contained only traces of N and P, and the syrup had a consistent N : P ratio of 4 : 1. The free acid was then made by again precipitating the Pb salt, which was washed and decomposed with H_2S. The filtrate from PbS was concentrated *in vacuo* to a small volume, filtered from the last traces of PbS, further concentrated, transferred to a basin, treated with alcohol and acetone, dried *in vacuo*, and ground to a fine powder. The yield of purified phosphopeptone was approx. 4 %. Its P content corresponded with approx. 25 % of the original casein. Inorganic phosphate liberated by trypsin corresponded with 11–12 %. The remaining 63 % not precipitated by lead acetate or alcohol awaits further investigation.

Dried *in vacuo* over $CaCl_2$, the purified caseo-phosphopeptone contained 10·56 % N and 5·77 % P. Hence N : P = 4 : 1. Amino-N determination showed 12·6 % of the total N. The phosphopeptone thus appears to be an octapeptide with 2 atoms of P.

Sulphur and diamino-acids are absent, tyrosine and tryptophan reactions are negative. Hydrolysis with 20 % HCl for 24 hr. raised the amino-N to 88 % of the total N, for 36 hr. to 98 %, and for 48 hr. to 100 %, which suggests a more resistant peptide linkage in the molecule, and shows the absence of cyclic N.

The solution of phosphopeptone is very acid in reaction:

0·2704 g. = 13·9 ml. *N*/10 NaOH to either phenol- or thymol-phthalein. On then adding neutralized formalin, the figure was raised to 15·7 ml. 0·2704 g. = 28·35 mg. N is thus equivalent to 1·39 mg. H and after formalin to 1·57 mg. H.

Ratio of acidic H to N atoms = 1·39 : 28·35/14 = 0·686/1 or 5·5/8
= 1·57 : 28·35/14 = 0·775/1 or 6·2/8.

The octapeptide would thus have six replaceable H atoms. Two will belong to the two H_3PO_4 radicles, two to 2 mol. glutamic acid (see below), one to a COOH group at the end of the chain, and the sixth to another COOH group.

Liberation of P by N/4 NaOH at 37°. For comparison with caseinogen which loses all its P as PO_4 ions in 24 hr. at 37°, 0·4240 g. phosphopeptone was dissolved in 100 ml. *N*/4 NaOH and placed in an incubator at 37°. Samples were taken at intervals and inorganic PO_4 precipitated as NH_4MgPO_4 and estimated by our usual procedure:

At 0 hr.	inorg. PO_4	= 0·421	mg. P	= 17·9	% hydrolysis.
4	,,	= 0·570	,,	= 24·3	,,
8	,,	= 1·566	,,	= 66·7	,,
12	,,	= 1·810	,,	= 77·1	,,
24	,,	= 2·040	,,	= 87·0	,,
48	,,	= 2·048	,,	= 87·3	,,
	Total P	= 2·346			

Rather more than half the P is separated in 8 hr. and the hydrolysis is not complete in 24 hr. and not increased in 48 hr. The difference in rate may be due to the position of the phosphoserine in the chain, one more easily hydrolysed in the middle portion, the other at the end of the chain.

The results contrast with the stability of phosphoserine (later publication) which is not hydrolysed by *N*/4 NaOH at 37° and at 100° loses 66 % in 48 hr.

Separation of H_3PO_4 by hydrolysis with acid. For future work on the isolation of amino-acids and confirmation of the production of a dipeptide of glutamic acid and phosphoserine hydrolysis by HCl at 100° was investigated:

With 5·5 *N* HCl for	24	hr.	inorg. PO_4 separated was	53·85	%	of total P
5·5	48			100		
2	10			15·4		

In comparison with phosphoserine which lost 60 % of its P as inorg. PO_4 by boiling with *N* HCl for 24 hr. the phosphopeptone is more stable.

Estimation of glutamic acid. The opportunity was taken of estimating glutamic acid by the micro-enzymic method of Cohen [1939] at the same time as in dephosphocasein and dephosphocaseose [Macara & Plimmer, 1940].

1 g. phosphopeptone was hydrolysed with 5·5 *N* HCl for 24 hr. The solution was evaporated *in vacuo* several times to remove HCl, the resulting syrup dissolved in water, neutralized to *p*H 3 and adjusted to 50 ml. A nitrogen estimation showed the concentration to be 2 %. The procedure as described by Macara & Plimmer was then carried out:

4 ml. hydrolysate contained	22·1	mg.	glutamic acid	=	27·6 %
5	25·9			=	25·9 %
				Mean	26·8 %

The mean value is 24·2 % of the total N, which indicates the presence of 1 mol. per 4 atoms of N or 2 mol. in 1 mol. of octapeptide.

Isolation of glutamic acid. 5 g. phosphopeptone were hydrolysed with 50 ml. 5·5 *N* HCl. The solution was concentrated *in vacuo* and saturated with dry HCl gas. Glutamic acid hydrochloride slowly crystallized out. Dried *in vacuo* the crude salt weighed 0·8 g. = 16 % of the phosphopeptone. The yield is far below the figure by estimation. Precipitation is probably inhibited by the presence of serine and its decomposition products.

Estimation of threonine. To establish the presence and determine the amount of this amino-acid the method of Block & Bolling [1939] as modified by Macara & Plimmer [1940] was used.

1·3824 g. phosphopeptone were hydrolysed with 5·5 *N* HCl for 36 hr. and the volume adjusted to 100 ml.; 2 ml. of this solution after treatment gave only a mere trace of colour with the reagent.

It had to be concluded that threonine was absent from the octapeptide.

Estimation of total β-hydroxyamino-acids. According to Van Slyke *et al.* [1940] oxidation of β-hydroxyamino-acids with KIO_4 completely separates the amino group as NH_3. The same hydrolysate was used as for threonine.

5 ml. gave	7·3	ml. *N*/14 NH_3	=	10·56	% total N
5 ml. gave	1·1	ml. *N*/14 NH_3 by aeration with Na_2CO_3	=	15	% of total N
and	1·2	ml. *N*/14 NH_3 on oxidation with KIO_4	=	16	% of total N

Assuming that amide groups are not present in the phosphopeptone and that the NH_3 liberated by Na_2CO_3 arises from decomposition of serine by boiling with HCl (see below) the total of 31 % of the total N indicates the presence of 2 mol. serine in the octapeptide if hydroxyglutamic is not present. Harington & Randall [1931] could not isolate this compound from caseinogen and Gulland & Morris [1934] obtained a doubtful 0·33 %.

Estimation of serine. Attempts to estimate serine by Rapoport's method [1937] were not successful; only brown charred solutions resulted [cf. Macara & Plimmer, 1940].

Liberation of NH_3 by hydrolysis. Posternak [1927, 1] noted the liberation of NH_3 during acid hydrolysis of phosphopeptone and regarded it as indicative of serine peptides and later [1928] found 12 % evolved from caseopeptone and 20 % from vitellopeptone. As higher amounts were evolved with greater concentrations of acid he considered it did not arise from amide groups. Serine under the same conditions gave 8 %. Hydrolysis of caseopeptone with NaOH gave higher amounts. Further examination of the liberation of NH_3 seemed advisable with serine, phosphoserine and caseopeptone.

Serine. (*a*) 0·5 g. serine was boiled under reflux with 60 ml. 5·5 *N* HCl. Samples were taken every 12 hr., made alkaline with Na_2CO_3 and the NH_3 aerated into standard acid.

(*b*) A similar experiment was made with the addition of 1 equiv. of H_3PO_4 to the solution.

With HCl only			With H_3PO_4		
After 12 hr.	NH_3 = 0·294 mg.	N = 2·7 % total N	= 0·294 mg.	N = 2·7 % total N	
24 ,,	= 0·399 ,,	= 4·0 ,,	= 0·370 ,,	= 3·3 ,,	
36 ,,	= 0·588 ,,	= 5·3 ,,	= 0·955 ,,	= 8·6 ,,	
60 ,,	= 0·785 ,,	= 6·7 ,,	= 1·920 ,,	= 17·1 ,,	

(*c*) Plimmer & Lowndes [1938] found 17·9 % N liberated on boiling serine for 6 hr. with 5*N* NaOH.

Phosphoserine. 0·11 g. was dissolved in 100 ml. 2*N* NaOH. Total N in 10 ml. = 0·7 mg. The remaining 90 ml. (= 6·3 mg.) diluted with 90 ml. H_2O were distilled and the NH_3 collected at 15 min. intervals in standard acid:

In 15 min.	NH_3 evolved	= 0·07 mg.
In next 15 min.	NH_3 evolved	= 0·07 mg.
,,	,,	= 0·07 mg.
After 75 min.	total	= 0·30 mg. = 4·76 % of total N

Caseopeptone. (*a*) As stated above under KIO_4 oxidation 15 % of the total N was evolved as NH_3 on boiling with 5·5 *N* HCl for 36 hr.

(*b*) 0·2694 g. was dissolved in 200 ml. *N* NaOH and distilled for 1 hr. The NH_3 evolved was 21·3 % of the total N.

(*c*) 0·3 g. was dissolved in 100 ml. 2*N* NaOH. 10 ml. were taken for total N = 3·55 mg. The remaining 90 ml. were diluted with 90 ml. H_2O and distilled and the NH_3 collected in standard acid and titrated at 15 min. intervals. The volume was kept constant by occasional addition of water.

After 15 min.	NH_3 = 5·00 mg.	N = 15·7 % of total N	(31·95 mg.)	
30 ,,	= 6·02 ,,	= 18·8 ,,	,,	,,
45 ,,	= 6·53 ,,	= 20·4 ,,	,,	,,
60 ,,	= 7·04 ,,	= 22·0 ,,	,,	,,
75 ,,	= 7·40 ,,	= 23·2 ,,	,,	,,
90 ,,	= 7·62 ,,	= 23·9 ,,	,,	,,
105 ,,	= 7·84 ,,	= 24·5 ,,	,,	,,
120 ,,	= 8·13 ,,	= 25·5 ,,	,,	,,

Serine and phosphoserine are comparatively stable to hydrolysis by acid and alkali. Caseopeptone is unstable to both. On plotting the figures of NH_3 liberation by alkali on a curve, the rapid evolution in the first 15 min. is equivalent to 1 atom of N out of the 8 atoms in the octapeptide (12·5 % of total N). A slower liberation follows up to 25·5 % in 120 min., which is equivalent to 2 atoms of N of the octapeptide. The difference in rate suggests loss of NH_3 from phosphoserine groups at the end or middle of the chain, a free amino group at the end being very labile.

Summary and discussion

Our preparation of caseo-phosphopeptone, judging from its N and amino-N contents, is an octapeptide containing two H_3PO_4 groups, 2 mol. glutamic acid and 2 mol. serine. Its acidity indicates the presence of another mol. of a dicarboxylic acid, leaving 3 mol. of simple amino-acids.

As laboratory work has been interrupted, calculations of molecular weight and deductions therefrom may be allowed:

From P content of 5·756 % and 2 atoms per mol.: mol. wt. is	$\frac{2\times31\times100}{5\cdot765}=1079$	
From glutamic acid content of 26·8 % and 2 mol.: mol. wt. is	$\frac{2\times147\times100}{26\cdot8}=1097$	
From NH_2-N figure of 1·33 %: mol. wt. is	$\frac{14\times100}{1\cdot33}=1053$	
	Mean = 1076	
Deduct 2 mol. glutamic acid 2×147	= 294	
2 mol. serine 2×105	= 210	
2 mol. H_3PO_4 2×98	= 196	
	700	
Less 2 ester linkages and 7 peptide linkages 9×18	= 162	
	538	538
Mol. wt. of remaining 4 mol.		= 538
Mean mol. wt. of amino-acids	= 134	
Mol. wt. of leucines	= 131	
Mol. wt. of aspartic acid	= 134	

The deductions are confirmatory of the isolation of 3 mol. of *iso*leucine by Posternak, and by Damodaran & Ramachandran. The latter authors find caseo-phosphopeptone to be a decapeptide with 3 mol. glutamic acid, 3 or 4 mol. serine and 3 mol. H_3PO_4. Rimington's phosphopeptone is a nona- or decapeptide. The isolation of a dipeptide of glutamic acid and phosphoserine by Levene & Hill points to the combination of these two amino-acids. In view of the rapid liberation of 1 mol. NH_3 by the action of $N/4$ NaOH and a difference in the rate of separation of H_3PO_4 we suggest that one phosphoserylglutamic group is at the end of the chain with a free amino group easily liberated, and the second group is in the middle of the chain, and picture the molecule of the octapeptide thus: phosphoserylglutamic-*X*-*X*-phosphoserylglutamic-*X*-*X*, where *X* is the unknown amino-acid, probably 3 mol. *iso*leucine and 1 mol. aspartic acid.

The difference between our octapeptide and the decapeptide seems to be 1 mol. of dipeptide of serine and glutamic acid, possibly at the other end of the chain from which it may be more easily split off: phosphoserylglutamic-*X*-*X*-phosphoserylglutamic-*X*-*X*-phosphoserylglutamic.

There is no evidence whether the dipeptide is phosphoserylglutamic acid, or glutamylphosphoserine. Both isomers may be present, but the one at the left-hand end seems to be phosphoserylglutamic acid. Phosphoserine may also be in combination with *iso*leucine or other amino-acid, if present.

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32. NOTE ON THE AMINO-ACIDS PRESENT IN PHOSPHOPEPTONE

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Some years ago Rimington & Kay [1926] gave the name 'phosphopeptone' to a substance present in tryptic digests of caseinogen and later isolated [Rimington, 1927, 1] by a process involving separation of inert material and then precipitation of the phosphorus-rich complex as an insoluble uranyl derivative and lastly as a copper salt.

The N/P ratio of the crude copper salt was approximately 3·3/1, but by fractional copper precipitation a main homogeneous constituent with N/P = 3·0 was obtained and used for detailed study of amino-acid composition, etc. The fractions with higher and lower N/P ratios were not analysed in this way, but it was realized that peptones of varying composition might easily be obtained as a result of more or less prolonged tryptic digestion before isolation was commenced. The main phosphopeptone was not completely stable towards activated pancreatic juice [Rimington & Kay, 1926].

Examination of phosphopeptone by the methods then available for the identification of amino-acids [Rimington, 1927, 2] revealed the presence of serine and a dicarboxylic acid considered to be hydroxyglutamic acid and also an incompletely identified substance thought, possibly, to be β-hydroxy-α-amino-butyric acid.

Since these early studies, investigations have been published by several workers. Schmidt [1934], after prolonged tryptic digestion of caseinogen, isolated a substance with N/P = 2, composed of glutamic acid and serine; fractions with higher N/P ratios were also encountered, when the digestion was less complete, in which a leucine was also present. Levene & Hill [1933], combining a preliminary tryptic digestion of caseinogen with subsequent hydrolysis by boiling acid, succeeded in isolating from the mixture a dipeptide of glutamic acid, serine and phosphoric acid, whilst Lipmann [1933] by boiling acid alone obtained from his hydrolysates serine phosphoric acid; peptides of higher N/P ratio were also present in the mixtures. More recently Damodaran & Ramachandran [1940], in a preliminary communication, claimed to have isolated a phosphorus-containing peptone from caseinogen by applying pepsin, separating the paranuclein formed and then digesting with trypsin. It had N/P = 3·3 and is reported to contain 3 mol. glutamic acid, 3 mol. *iso*leucine and 4 mol. serine.

Although it is only to be expected that the use of different chemical or enzymic hydrolyses (and for different lengths of time) should lead to phosphorus-containing materials of different constitution, it was thought desirable to re-examine the original 'phosphopeptone' preparations, now that more reliable methods are available for the detection and determination of amino-acids like threonine. The isolation of serine phosphoric acid after acid hydrolysis of caseinogen by Levene & Hill [1933] supports my conclusion that a part, at least, of the phosphorus in phosphopeptone is united to serine, but from the colorimetric determinations presented by Rapoport [1937] the question arises whether or not all the phosphorus in caseinogen is bound in this way.

Experimental

There was available for this work a small quantity of the original phosphopeptone described in 1927 and a larger quantity of crude copper salt prepared subsequently. From the latter the copper was removed as sulphide, employing fuller's earth to facilitate filtration [Rimington, 1927, 1], and the free peptone was precipitated by addition of excess of alcohol. After dehydration with absolute alcohol and ether it was dried *in vacuo* at 105° over P_2O_5. Both peptones were colourless powders, readily soluble in water to form strongly acid solutions.

Analysis. Determination of N/P ratios gave the values 3·01/1 and 3·33/1 for the original peptone and the larger sample respectively. Since amino-N determinations and other data indicate that there are 3 P atoms per mol., these substances contained 9 and 10 N atoms respectively and will be referred to as the N_9 and N_{10} peptones. Elementary analysis gave the following results:

	C	H	N	P
N_9 peptone	35·29	6·16	9·86	7·27
$C_{37}H_{60}O_{33}N_9P_3$ requires	35·50	4·80	10·07	7·44
N_{10} peptone	37·58	6·16	10·40	6·88
$C_{43}H_{71}O_{34}N_{10}P_3$ requires	37·83	5·21	10·27	6·82

It will be noticed that the empirical formulae, deduced from the analytical figures and assuming 3 phosphoric acid residues per mol., differ by one molecule of leucine or *iso*leucine, $C_6H_{13}O_2N$, in peptide linkage.

Determination of dicarboxylic acids and isolation of glutamic acid

Hydrolysates of the N_{10} and N_9 peptones were prepared by boiling for 48 hr. with 20 % HCl. Portions of these were worked up by Foreman's quantitative calcium salt method for the determination of dicarboxylic amino-acids. The result in each case corresponded to rather less than 4 mol. of dicarboxylic acid per mol. of peptone, thus:

N_9 peptone:	total N in hydrolysate	19·72 mg.
	4/9 of total N	8·76 ,,
	total N in Ca salt fraction	8·64 ,,
N_{10} peptone:	total N in hydrolysate	166·0 ,,
	4/10 of total N	66·4 ,,
	total N in Ca salt fraction	59·3 ,,

Since hydroxyglutamic acid had previously been suspected, in the dicarboxylic acid fraction, calcium was removed from a portion by means of oxalic acid and the crude amino-acid obtained oxidized with chloramine T as described by Harington & Randall [1931]. The mixture was treated with *p*-nitrophenylhydrazine and warmed on the water bath for 45 min., then filtered and the residue washed repeatedly with boiling methyl alcohol until a small dark red residue remained, a trace of which afforded an intense pure blue colour with alcoholic KOH. The substance was recrystallized from hot nitrobenzene, from which it separated in the form of dark red needles melting at 322–7°. The yield was only 4·4 mg. from 0·3 g. of starting material and the substance contained 22·6 % N. It was considered to be the *p*-nitrophenylosazone of glyoxal, since the M.P. was not lowered by admixture with this substance (N = 25·6 %). Mixed with the corresponding derivative of malic semi-aldehyde (N = 21·7 %), kindly supplied by Prof. Harington, the M.P. was depressed approximately 20°. Unfortunately the M.P. of these substances are not very suitable for their

characterization; however, since serine is known to be unstable under the hydrolytic conditions employed and could readily yield glyoxal, the conclusion is probably justified that serine was present as an impurity in the crude dicarboxylic acid fraction examined.

Further evidence against the presence of any α-amino-β-hydroxy-acid in this fraction was obtained by treating an aliquot portion with periodic acid [Van Slyke *et al.* 1940], which failed to liberate any ammonia.

The remainder of the crude dicarboxylic fraction was freed from calcium by oxalic acid and concentrated to a small volume. Crystals separated which were filtered off, washed sparingly and dried (crop 1) and, by further concentration of the mother liquors, a second deposit (crop 2) was obtained. The final mother liquors were treated with several volumes of alcohol, when a small amount of crystalline material (crop 3) slowly separated. These fractions were analysed with the following result:

	C	H	N
Crop 1 (M.P. 198°)	41·01	6·05	9·64
Crop 2	40·59	6·28	9·50
Crop 3 (corrected for ash)	36·45	6·49	—
Glutamic acid requires	40·85	6·17	9·52
Hydroxyglutamic acid requires	36·81	5·52	8·59

It would appear, in the light of the experiments already recorded, that crop 3 consisted only of impure glutamic acid. There was unfortunately insufficient material for N determination. In view of the similar C and H figures which were previously reported [Rimington, 1927, 2] for the dicarboxylic acid fraction of phosphopeptone, it is noteworthy that impure glutamic acid may give analytical results so closely approaching those required by hydroxyglutamic acid.[1]

The optical rotatory power of the material of crop 1 in 10 % HCl was $[\alpha]_{5461} = +37{\cdot}39°$. The hydrochloride was easily prepared in 88 % yield and, dissolved in water (3·2 %), had $[\alpha]_D = +22{\cdot}04°$, $[\alpha]_{5461} = +27{\cdot}11°$. Anslow & King [1927] give $[\alpha]_{5461}$ as 26·85° for a 2·5 % aqueous solution and 27·45° for a 5 % solution.

Past attempts to isolate glutamic acid as the hydrochloride directly from phosphopeptone hydrolysates had not succeeded and the separation has now only been achieved with difficulty and in poor yield. It would appear that the other products of hydrolysis, such as serine and phosphoric acid, exert an inhibiting effect upon the crystallization of the hydrochloride.

5 g. of N_{10} peptone were refluxed with 50 ml. of 20 % HCl for 30 hr. and the mixture then saturated with HCl gas. In spite of seeding, not more than a trace of material separated after 24 hr. at 0°. The liquid was therefore concentrated to small volume and again saturated with HCl, when 1·4 g. of crystals separated in the course of several days. Repeated concentration and saturation raised the yield to 1·48 g., which represents only between 2 and 3 mol. per mol. of peptone.

It must be concluded from the above findings that glutamic acid and not hydroxyglutamic acid is present in phosphopeptone.

Isolation and identification of isoleucine *from N_{10} peptone*

The filtrate from the calcium salts of a hydrolysate corresponding to 1·6 g. of N_{10} peptone was freed from calcium by oxalic acid, made slightly alkaline to litmus with ammonia and concentrated to about 2·5 ml. Upon cooling in the

[1] Prof. A. C. Chibnall informs me that he has often obtained analytical figures for impure glutamic and aspartic acids which correspond closely to those required for hydroxyglutamic acid.

ice-chest, crystals separated which, after recrystallization from 75 % alcohol, formed fragile glistening plates. Concentration of the mother liquor yielded a further small crop: total yield 73·5 mg. 1 mol. per mol. of peptone corresponds to 155 mg.

	C	H	N
Analysis: Found	54·88	9·98	10·73
*iso*Leucine ($C_6H_{13}O_2N$) requires	54·97	9·99	10·69

A 1·6 % solution in 20 % HCl had $[\alpha]_D = +34·38°$; $[\alpha]_{5461} = +40·62°$. The $[\alpha]_D$ of *iso*leucine in this solvent is +34·26° and that of leucine is +15·4°. For further identification, the phenylisocyanate derivative was prepared. It had M.P. 117°; that of *iso*leucine melts at 119°.

Examination for threonine

Block & Bolling [1939] have recently described a method for the micro-determination of threonine depending upon its oxidation with lead tetraacetate to acetaldehyde which is determined colorimetrically after reaction with *p*-hydroxydiphenyl; serine does not interfere. When this method was applied to a phosphopeptone hydrolysate only a trace of colour resulted, corresponding to less than 1/40 mol. threonine per mol. of peptone. It is therefore concluded that threonine is absent from phosphopeptone.

Determination of serine and total hydroxyamino-acids

Phosphoserine is remarkably resistant to hydrolysis with boiling acids (personal communication from Prof. R. H. A. Plimmer); this property evidently made possible the isolation of phosphoserine by Lipmann [1933] and by Schmidt [1934] from acid hydrolysates of caseinogen. Serine itself, however, is readily transformed by hot mineral acids into ammonia, glyceric acid, glyoxal and other products (compare Damodaran & Ramachandran [1940]), so that one is beset with the difficulty, in attempting its determination in phosphopeptone, of achieving complete liberation from phosphoric acid with the minimum of destruction. The use of alkali for this purpose was not satisfactory.

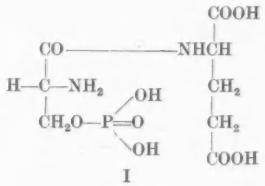
2 g. of N_{10} peptone were refluxed with 20 % HCl for 160 hr., by which time all the phosphorus was present as free phosphoric acid. Direct determination of ammonia by an aeration method showed 26·5 % of the total N to be present in this form. A periodate oxidation liberated another 13·6 %, making in all 40·1 % of the total N, or 4 mol. per mol. of peptone, derivable from hydroxy-amino-acids. On a portion of the hydrolysate, serine was determined by Rapoport's [1937] colorimetric method. The amount present corresponded to only 5·6 % of the total N, in contrast to the figure of 13·6 % of hydroxyamino-acid by periodate oxidation recorded above. This finding is similar to Rapoport's own results with casein hydrolysates, from which he concluded that at least one-third of the total phosphorus of the protein was bound to hydroxyamino-acids other than serine. In view of the known instability of serine under the conditions of hydrolysis it is doubtful, however, if much emphasis should be laid upon such negative data. It is possible that a portion of the serine originally present has been so changed that it is no longer capable of yielding glyceric acid. In fact, Damodaran & Ramachandran [1940] report that they found both lactic and pyruvic acids, presumably derived from serine, in their phosphopeptone hydrolysates.

Nature of the phosphorus linkage in phosphopeptone

The suggestion was made by Rimington [1927, 2] that two of the three phosphoric acid residues of phosphopeptone were held in mono-ester linkage whilst

the third was in the form of a di-ester. This suggestion was based upon the observed difference in action of bone and kidney phosphatases upon phosphopeptone, the former liberating only two-thirds of the phosphorus as free phosphoric acid, the latter producing complete hydrolysis. Since phosphoserine is relatively resistant to hydrolysis by boiling dilute alkali, whereas the phosphorus is rapidly split off as phosphoric acid from caseinogen by 1 % NaOH at 37°, another explanation appears possible. The action of alkali upon phosphopeptone was therefore studied again in detail.

It was found that only about two-thirds of the total phosphorus was removed as phosphoric acid by 1 % NaOH at 37° and the time curve of the hydrolysis bore a strong resemblance to that recording the action of bone phosphatase upon phosphopeptone. It appears that the relative stability towards alkali of phosphoserine, or of a terminal phosphoserine residue in peptone linkage with other amino-acids as in phosphopeptone, may be conditioned by the close proximity of the free amino group. Phosphocholine, which as Beznák & Chain [1937] point out, has a betaine structure, was similarly found by them to be remarkably stable towards boiling acids and alkalis although hydrolysed by both bone and kidney phosphatases. Plimmer & Burch [1937] reported the same behaviour on the part of phosphoaminoethanol and phosphocholine. It would be very interesting in this connexion to discover whether Levene & Hill's [1933] phosphodipeptide possessed structure I or II (Levene & Hill have formulated their substance as an aspartic acid derivative).



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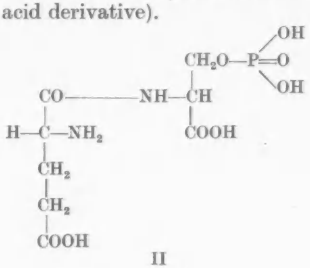

II

Discussion

On the assumption that only glutamic acid, *iso*leucine, serine and phosphoric acid are present in the N_{10} phosphopeptone, it is found possible to construct the following formulae, which are in good agreement with the elementary analysis of the peptone and roughly correspond with the yields of each amino-acid experimentally determined.

N_{10} peptone:

$$5\ \text{glutamic acid} + 1\ \textit{iso}\text{leucine} + 4\ \text{serine} + 3H_3PO_4 - 12H_2O = C_{43}H_{71}O_{34}N_{10}P_3,$$

requiring	37·83 % C;	5·21 % H;	10·27 % N;	6·82 % P.
Found	37·58 % C;	6·16 % H;	10·40 % N;	6·88 % P.

Experimentally not more than 4 mol. of glutamic acid were found by the Ca salt method, but there were also found 4 mol. of serine and at least 1 mol. of *iso*-leucine. Similarly for the

N_9 peptone:

$$5\ \text{glutamic acid} + 4\ \text{serine} + 3H_3PO_4 - 11H_2O = C_{77}H_{60}O_{33}N_9P_3,$$

requiring	35·50 % C;	4·80 % H;	10·07 % N;	7·44 % P.
Found	35·38 % C;	5·14 % H;	10·03 % N;	7·41 % P.

Once more the glutamic acid determination fell short of the required figure, but the difference in elementary composition between the N_{10} and N_9 peptones corresponds to exactly 1 mol. of *iso*leucine, and it will be remembered that this amino-acid was not detected in the course of the original study made of the N_9 phosphopeptone [Rimington, 1927, 2].

Whilst, then, some reserve must still be exercised, it is felt that the compositions suggested above do probably represent the true nature of the two peptones which have been here subjected to study. Naturally, other conditions of preparation of the caseinogen hydrolysate, e.g. prolonged tryptic digestion, might lead to peptones with a different composition, as the work of Levene & Hill and others clearly indicates; most drastic hydrolysis leads to the comparatively stable phosphoserine.

Damodaran & Ramachandran [1940] considered the phosphopeptone which they prepared to be composed of 3 mol. of glutamic acid, 3 mol. of *iso*leucine, 4 mol. of serine and 3 mol. of phosphoric acid. The yields of these substances which they obtained were in fairly good agreement with such a conclusion, but no elementary analysis of the complete peptone is recorded. This is unfortunate, since elementary analyses would have afforded a check upon the conclusions arrived at. The composition demanded by such an assembly of amino-acids differs considerably from that determined experimentally for my N_{10} peptone, thus:

N_{10} peptone:	C	H	N	P
Found	37·78	5·30	10·25	6·81
Calculated formula $C_{43}H_{72}O_{34}N_{10}P_3$ requires	37·83	5·21	10·27	6·82
Damodaran & Ramachandran's peptone would require	40·54	5·93	10·51	6·98

One must conclude that Damodaran & Ramachandran's peptone, prepared as it is from paranuclein, differs from the phosphopeptone prepared according to my method by the action of trypsin only upon caseinogen. It appears that the essential phosphorus linkage in caseinogen is between phosphoric acid and serine and that this amino-acid is united in peptide linkage with other amino-acids, predominantly glutamic acid but possibly also *iso*leucine, and that tryptic digestion rapidly sets free these complexes from the rest of the protein molecule (compare the evidence of Rimington & Kay [1926]), but that they themselves being more resistant towards enzymic hydrolysis are only slowly degraded into peptones or peptides of ever decreasing complexity. A phosphopeptone unit containing 10 amino-acids and 3 mol. of phosphoric acid is comparatively readily isolated.

This reinvestigation of phosphopeptone is a result of communications made to the Biochemical Society (*Chem. and Ind.* **59**, 185, 1940) by Prof. R. H. A. Plimmer on 'Phosphoryl hydroxyamino-acids' and by Dr J. Lowndes on 'The composition of phosphopeptone'. I have enjoyed the benefit of frequent discussion with the authors and this paper is published with their approval. I desire to thank Prof. Plimmer for a specimen of *dl*-threonine and Prof. Robison for a specimen of the Ba salt of 3-phosphoglyceric acid.

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33. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

2. FORMATION AND PROPERTIES OF CHOLEGLOBIN[1]

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In a preliminary publication [Lemberg, Legge & Lockwood, 1938] we have reported that by the coupled oxidation of haemoglobin and ascorbic acid the prosthetic group of haemoglobin is oxidized. Under appropriate conditions a product, still mixed with haemoglobin, can be obtained, in which the new prosthetic group is combined with native globin. Acids easily split off iron from the product of oxidation and form bile pigments. These observations suggest that the compound is a bile pigment-iron-native globin complex, which we have called choleglobin.

In Part 1 of this series [Lemberg, Legge & Lockwood, 1939] we have discussed the initial mechanism of the reaction. In the present paper we describe in detail the preparation and properties of choleglobin, and we compare it with related substances which also contain easily detachable iron and yield bile pigment on acid splitting. Such substances are verdohaemochromogen obtained from pyridine-haemochromogen by coupled oxidation with ascorbic acid [Lemberg, Cortis-Jones & Norrie, 1938], and the 'pseudohaemoglobin' obtained by Barkan & Schales [1937; 1938] by the action of hydrogen peroxide on haemoglobin in the presence of cyanide.

Experimental

Preparation of choleglobin. We have used solutions of crystalline horse oxyhaemoglobin prepared by the method of Heidelberger [1922], and also washed erythrocytes of sheep, ox and horse, laked by freezing and freed from stroma by adjusting the *p*H to 5·8 and keeping in the refrigerator. All these solutions behaved qualitatively in the same way.

At first the solutions were Seitz-filtered and the experiments carried out under strictly sterile conditions, but this precaution was later found to be unnecessary for experiments up to 2 hr. incubation time. No signs of bacterial contamination were observed and the rate of formation of the choleglobin absorption band was the same as in experiments under sterile conditions.

The haemoglobin concentration varied between 0·3 and 3%, the majority of experiments being carried out with a haemoglobin solution of about 0·7%. Crystalline ascorbic acid (B.D.H.) was used. Its concentration in the reaction mixture was usually 100 mg. per 100 ml. The solutions were buffered with phosphate; phosphate is not necessary for the reaction with ascorbic acid, choleglobin being formed without the addition of buffers, or with borate buffers. The

[1] The work was carried out under a grant from the National Health and Medical Research Council of Australia.

majority of experiments were carried out either at *p*H 7·2, which was obtained by adding 20 ml. of *M*/20 phosphate buffer *p*H 6·6 to 30 ml. of a 1·2% haemoglobin solution, or at *p*H 8·5 obtained by adding 5 ml. of a solution of disodium hydrogen phosphate saturated at room temperature (20°) to 45 ml. of an 0·8% haemoglobin solution. The final haemoglobin concentration was thus 0·72%.

The reaction mixtures were incubated at 37° in flat culture flasks exposed to atmospheric oxygen without shaking in a layer of 4 mm. depth. A few experiments were carried out in Warburg vessels with shaking.

Spectroscopic observations

Choleglobin. Under these conditions the absorption band of reduced choleglobin at 628–630 mμ appears a few minutes after the reaction has been started by the addition of ascorbic acid. There was no noticeable formation of reduced haemoglobin and the bands of oxyhaemoglobin remained visible. The band in the orange can be due neither to methaemoglobin nor to methaemalbumin [Fairley, 1938; 1939], since addition of $Na_2S_2O_4$ does not destroy it, but increases its strength. Alkali shifts the band to 618–622 mμ, transforming choleglobin into denatured globin-cholehaemochromogen. If pyridine is added to the choleglobin solution until the precipitate of denatured globin-haemochromogens is redissolved and $Na_2S_2O_4$ added at once, there is also only the band of cholehaemochromogen at 618–620 mμ beside the bands of protohaemochromogen in the green. $Na_2S_2O_4$ must be added without delay in order to stop the formation of verdohaemochromogen from pyridine protohaemochromogen by coupled oxidation with the excess of ascorbic acid.

During the incubation the absorption band of reduced choleglobin gradually increases in intensity, but later, after about 30 min. of incubation, it is replaced by an absorption band at about 670 mμ. On reduction with $Na_2S_2O_4$ this band gives place to the band of reduced choleglobin at 630 mμ; it is therefore due either to ferric choleglobin or to oxycholeglobin. Carbon monoxide also causes the disappearance of the band at 670 mμ and the formation of a strong band at 628 mμ. The position of this band is very close to that of reduced choleglobin and may lead to the assumption that CO acts only as an inert gas allowing the excess ascorbic acid to reduce the oxidized choleglobin. It can, however, be proved that a CO-choleglobin is formed. Shaking with a mixture of 20% O_2 and 80% CO causes the band at 670 mμ to be replaced by that at 628 mμ, while shaking with air leaves the band at 670 mμ unaltered. CO also reacts with the alkaline solution of cholehaemochromogen shifting the absorption band from 618 mμ to 628 mμ. CO-choleglobin and CO-cholehaemochromogen thus have their absorption bands in the same position, just as do CO-haemoglobin and CO-protohaemochromogen. In the chole-series, however, the CO-compound has an absorption very similar to that of the reduced choleglobin, while in the proto-series the bands of the CO-compound differ widely from that of reduced haemoglobin.

These observations do not offer sufficient proof that the oxidized form of choleglobin is oxycholeglobin with O_2 reversibly bound to ferrous choleglobin. Ascorbic acid is still present in the solution and CO may not react directly with the oxidized compound, but by combining with ferrous choleglobin it may shift an equilibrium between ferric and ferrous forms, normally much in favour of the ferric form, in favour of the ferrous form, until the formation of CO-choleglobin is practically complete. Experiments carried out in order to decide whether the oxidized form of choleglobin is ferric or contains O_2 reversibly bound, will be described later.

Cholehaemochromogen. After about 45 min. incubation a green precipitate begins to form as a bottom layer and as a thin surface skin. We shall call this precipitate 'green pigment'; it has been previously described by Anderson & Hart [1934] and by Edlbacher & v. Segesser [1937]. After 80 min., more green pigment has been formed which, up to this stage, is almost free from protohaemochromogen. It is insoluble in water, soluble in dilute alkali, 1 % acetic acid or pyridine. These solutions are olive-green. On reduction with $Na_2S_2O_4$ they turn pure green, but seen against artificial light the colour is brown. The absorption spectrum of the reduced alkaline solution shows, beside weak protohaemochromogen bands, the band of ferrous cholehaemochromogen at 616–618 mμ, which is shifted by CO to 628 mμ. In pyridine the band lies at 619 mμ, while in dilute acetic acid or in neutral aqueous suspension the band is found at 628 mμ. Green pigment consists thus mainly of denatured globin choleparahaematin (ferric cholehaemochromogen). The absorption spectrum of the unreduced ferric compound varies with the conditions. In pyridine there is a broad band with its centre at about 612 mμ and a second band at 665 mμ. This second band is not that of verdohaemochromogen, since it is abolished by $Na_2S_2O_4$ reduction, while the similar absorption band of verdohaemochromogen is not altered by $Na_2S_2O_4$ in neutral pyridine solution. In 1 % Na_2CO_3 there is only an indistinct absorption band at about 595 mμ, while in *N*/15 NaOH a sharp band at 646·5 mμ appears. The solution in dilute acetic acid displays an absorption band at about 610 mμ.

Although the absorption spectrum of ferrous cholehaemochromogen in neutral suspension or in weakly acid solution does not differ from that of choleglobin, the two compounds differ totally in solubility. Cholehaemochromogen is insoluble at neutral reaction and is quantitatively precipitated from its alkaline or acid solutions by neutralization or by dialysis against distilled water. Choleglobin is soluble at neutral reaction and this solubility is not due to the protective colloid action of admixed haemoglobin. A haemoglobin solution of *p*H 7·2 or 8·5 does not dissolve green pigment; this can be quantitatively removed by filtration and no choleglobin band is observed in the filtrate. Choleglobin, however, can be redissolved in water after precipitation with ammonium sulphate, and can be absorbed on aluminium hydroxide and eluted from it by a phosphate buffer of *p*H 8·5.

Denaturation of choleglobin. It is of interest that there is no denaturation of oxyhaemoglobin under the conditions of our experiment, if ascorbic acid is absent. Two explanations of the denaturation leading to green pigment are possible. Either choleglobin is less stable than haemoglobin and undergoes spontaneous denaturation similar to that of free native globin, or the denaturation is due to an oxidation of the globin part of the haemoglobin molecule accompanying the oxidation of the prosthetic group. After the removal of ascorbic acid, choleglobin, though undergoing secondary changes to be described later, does not appear to be very readily denatured. It might be assumed, however, that the replacement of the four groups of haemoglobin by cholehaem groups does not occur simultaneously and that choleglobins with one or two bile pigment-haem groups are stable, while a choleglobin with all four haem groups replaced by cholehaem would undergo spontaneous denaturation. We have recently shown [Lemberg, Norrie & Legge, 1939] that catalase is probably a compound with three protohaematin and one bile pigment-haematin group per molecule. We were indeed, unable to obtain a choleglobin solution with more than 60 % cholehaematin and less than 40 % of protohaematin.

However this may be, the second explanation certainly also holds. Holden and his collaborators [Holden, 1936; 1937; Rawlinson, 1939] have repeatedly

described oxidative denaturations of haemoglobin. Lemberg & Legge [1938] have shown that a part of the oxygen evolved from oxyhaemoglobin by acids is taken up through oxidation of the protein part of the molecule. While up to 80 min. incubation with ascorbic acid there is little denaturation of haemoglobin, after 2 hr. the precipitate contains a considerable proportion of ferric denatured globin-protohaemochromogen (cathaemoglobin), and the longer the reaction proceeds, the more haemoglobin is denatured. There appear to be also further changes in the nature of the protein in the precipitate; its solubility in alkali and pyridine decreases. Finally, as will be described in the fifth paper of this series, the prosthetic groups, both protohaem and cholehaem, are bound to the protein of the precipitate much more firmly than in the choleglobin solution or in the haemochromogens produced from it by alkali denaturation.

These observations enhance the steadily increasing amount of evidence that the products obtained by 'denaturing' one and the same protein by different methods may differ considerably (cf. the discussion of this problem in the *Cold Spring Harbor Symposia on Quantitative Biology* [1938]).

Products of the later stages of the reaction and of further oxidation of cholehaemochromogen

After incubation for several hours there is a great deal of precipitate, now more brown than green. The supernatant solution contains oxidized choleglobin, but the band at 670 mμ no longer disappears completely on reduction with $Na_2S_2O_4$ or with CO. On addition of pyridine and $Na_2S_2O_4$ an absorption band at 665 mμ, in addition to the cholehaemochromogen band at 618 mμ, appears even if $Na_2S_2O_4$ is added at once to the pyridine solution. The compound which causes the band at 665 mμ may be verdohaemochromogen, and verdoglobin[1] may be present in the supernatant solution before addition of pyridine. Addition of alkali to the reduced solution decreases the strength of the band in the red, and reduced verdoglobin behaves in a similar way, being transformed into a yellow compound. The strength of the cholehaemochromogen band at 618 mμ appears, however, to be increased by the addition of alkali; verdoglobin is not transformed into cholehaemochromogen by alkali, and if the increase of the band at 618 mμ accompanying the decrease of the band in the red is due to a transformation of the compound causing this band into cholehaemochromogen, the compound cannot be verdoglobin.

A substance with a similar absorption band in the red is produced if a pyridine solution of 'green pigment', which contains practically no protohaemochromogen, is left standing in the presence of air and ascorbic acid. Brown solutions are obtained which on reduction with $Na_2S_2O_4$ no longer display the absorption band of cholehaemochromogen, but a band at 657 mμ and a weaker band at 534 mμ. This compound is not formed if the ascorbic acid is washed away from the 'green pigment' before dissolving it in pyridine, or if the solution is left standing *in vacuo*. There can be no doubt, therefore, that the new compound is an oxidation product of cholehaemochromogen, formed from it by coupled oxidation with ascorbic acid in pyridine. In its absorption spectrum it resembles verdohaemochromogen and also in its behaviour towards $Na_2S_2O_4$ in alkaline solution. Nevertheless its identity with verdohaemochromogen is doubtful. The latter is green and gives rise to yellow compounds only by the action of $Na_2S_2O_4$ in alkali, while the colour of the oxidation product from cholehaemochromogen is brown in the ferric state as well as when reduced in neutral pyridine solution.

[1] Cf. Lemberg & Wyndham [1937, p. 352]. We prefer now to call the compound of verdohaem with native globin verdoglobin in spite of the fact that it does not reversibly unite with oxygen.

Absorption curves

The absorption curves of choleglobin and its derivatives were investigated with a Visible Absorption Spectrophotometer of Adam Hilger, consisting of the Barfit Hilger Constant Deviation Wavelength Spectrometer with prism n_D 1·62 and the Barfit Hilger Nutting Photometer on the Standard Accessory Bar.

Fig. 1 shows the absorption curves in the region from 740 to 600 mμ of the oxygenated choleglobin solution (full-drawn line), that of the same solution after reduction with $Na_2S_2O_4$ (dotted line) and again after saturation with coal gas (interrupted line). The absorption curves in the region of 600–500 mμ were mainly those of the accompanying haemoglobin compounds, the maximal

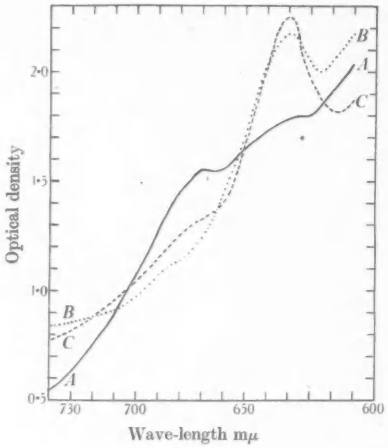

Fig. 1.

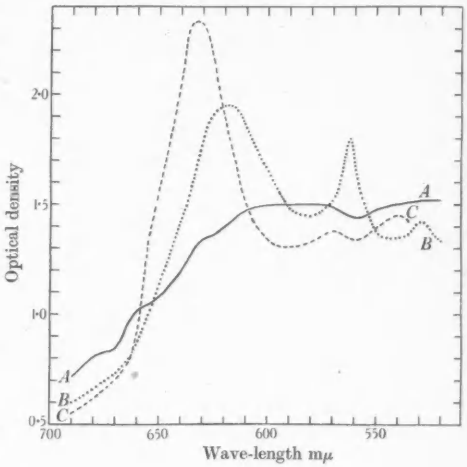

Fig. 2.

Fig. 1. Choleglobin. Curve *A* (full line), oxygenated. Curve *B* (dotted line), reduced. Curve *C* (interrupted line), CO.

Fig. 2. Cholehaemochromogen. Curve *A* (full line), green pigment in 1% Na_2CO_3. Curve *B* (dotted line), reduced with $Na_2S_2O_4$. Curve *C* (interrupted line), reduced with $Na_2S_2O_4$ in coal gas.

density of the CO-haemoglobin at 570 mμ being 1·63 times as strong as that of the CO-choleglobin at 630 mμ. The absorption band of the oxygenated solution at 670 mμ is far less marked than when viewed through a pocket spectroscope. Reduced choleglobin and CO-choleglobin both show a strong absorption band at 630 mμ, that of the CO-compound being somewhat sharper. Their absorptions in the wave-length region of 700–655 mμ are markedly less than that of oxygenated choleglobin.

The absorption curves of some cholehaemochromogen derivatives are given in Fig. 2. (The fully drawn line represents the absorption curve of green pigment dissolved in 1 % Na_2CO_3, the dotted line that of the ferrous cholehaemochromogen obtained by reduction with $Na_2S_2O_4$.) CO without reducer has no effect on the absorption curve of ferric cholehaemochromogen, but in the presence of $Na_2S_2O_4$ the band of the ferrous CO-cholehaemochromogen at 628 mμ appears which is stronger and sharper than that of the CO-free compound. The opposite

is true with regard to the bands of protohaemochromogen at 559 mμ and of CO-protohaemochromogen at 570 mμ.

From the appearance of these maxima in the absorption curves of 'green pigment' derivatives it is evident that green pigment contains some protohaemochromogen. The ratio $\epsilon_{630} : \epsilon_{570}$ of its CO-haemochromogen was 1·70. We have obtained other preparations with a higher ratio, the highest being 2·08. The last-mentioned preparation gave only a just noticeable hump at 560 mμ due to ferrous protohaemochromogen. If we assume as a first approximation: ϵ_{570} of cholehaemochromogen $= \epsilon_{570}$ of CO-cholehaemochromogen $= \epsilon_{557}$ of cholehaemochromogen $= \epsilon_{557}$ of CO-cholehaemochromogen $= x$, and ϵ_{570} of CO-protohaemochromogen $= y$, we have two equations with two unknowns:

$$\epsilon^{CO}_{570} = x + y, \quad \epsilon_{557} = x + 2{\cdot}14y,$$

the factor 2·14 being the measured ratio ϵ_{557} of protohaemochromogen to ϵ_{570} of CO-protohaemochromogen. If we substitute the values of ϵ^{CO}_{570} and ϵ_{557} from the curves in Fig. 2, we obtain $x = 0{\cdot}99$ and $\epsilon_{630} : \epsilon_{570}$ of CO-cholehaemochromogen $= 2{\cdot}33 : 0{\cdot}99 = 2{\cdot}35$. From this figure and the highest figure actually observed, we may safely conclude that 2·22 for this ratio is a good approximation; this figure will be used in Part 3 for establishing the absolute absorption values of choleglobin and cholehaemochromogen.

Does choleglobin combine reversibly with oxygen?

The question whether or not choleglobin unites reversibly with oxygen has an important bearing on the problem of the 'labile iron' in blood which we shall discuss in Part 4. We have therefore studied it in some detail.

A solution of choleglobin, obtained by 90 min. incubation of oxyhaemoglobin with 100 mg. per 100 ml. ascorbic acid at *p*H 8·5 under the conditions described above, was left at room temperature for 16 hr. in order to complete the oxidation of ascorbic acid, and then filtered. This solution failed to show any oxygen uptake in a Warburg respirometer during 10 min., but still contained small amounts of indophenol-reducing substance in the trichloroacetic acid filtrate (less than 3 mg. ascorbic acid per 100 ml.). Fig. 3 shows the effect of coal gas with acid but without added $Na_2S_2O_4$ on the absorption curve of the choleglobin (oxygenated solution fully drawn line). CO without reducer (interrupted line) still causes the appearance of a distinct absorption maximum at 630 mμ and a decrease of absorption at 670 mμ, but the effect of CO plus reducer is more marked. Similar results were obtained with a solution of choleglobin dialysed for 16 hr. against distilled water. On exhaustion of a solution of CO-choleglobin in a Thunberg tube, followed by reoxygenation, the band at 630 mμ disappeared and was again replaced by the band at 670 mμ. These observations lend some support

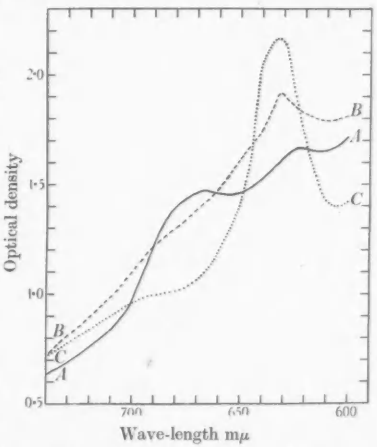

Fig. 3. Reaction of choleglobin with carbon monoxide. Curve *A* (full line), oxygenated. Curve *B* (interrupted lines), in coal gas. Curve *C* (dotted line), in coal gas with $Na_2S_2O_4$.

to the assumption that the oxygenated form of choleglobin reacts with CO and is, therefore, ferrous oxycholeglobin, with oxygen bound reversibly as in oxyhaemoglobin. The presence of small amounts of reducing substances in the solution does not, however, allow a definite conclusion.

We have attempted to remove the last traces of reducing substances by prolonged dialysis or by precipitation with ammonium sulphate followed by careful washing of the precipitate with saturated ammonium sulphate solution. Solutions were thus obtained which no longer reacted with CO without $Na_2S_2O_4$ (Fig. 4). The oxygenated solution, however, failed now to show the typical absorption band at 670 mμ of the original oxygenated choleglobin. During the dialysis some methaemoglobin was formed from oxyhaemoglobin, and if oxycholeglobin exists,

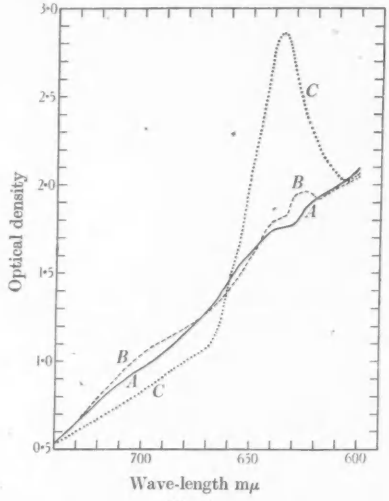

Fig. 4.

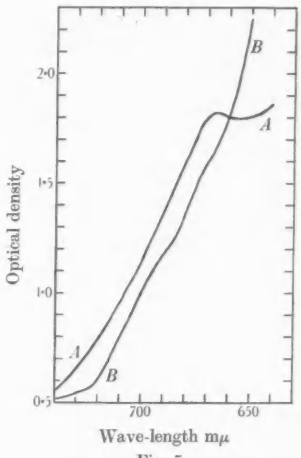

Fig. 5.

Fig. 4. Dialysed choleglobin and carbon monoxide. Curve *A* (full line), oxygenated. Curve *B* (interrupted line), in coal gas. Curve *C* (dotted line), in coal gas with $Na_2S_2O_4$.

Fig. 5. Effect of ferricyanide on oxygenated choleglobin. Curve *A*, oxygenated choleglobin. Curve *B*, the same after addition of ferricyanide.

it may be more easily transformed into a metacholeglobin than oxyhaemoglobin into methaemoglobin and thus lose its property of combining with CO without $Na_2S_2O_4$. This view is supported by the observation that ferricyanide destroyed the band at 670 mμ and decreased the absorption in this wave-length region (Fig. 5), but we failed to restore the band at 670 mμ by reduction of the ferricyanide-treated solution with an amount of $Na_2S_2O_4$ just sufficient to reduce the accompanying methaemoglobin to reduced haemoglobin and subsequent oxygenation; oxyhaemoglobin could be thus reconstituted.

Evacuation of a fresh choleglobin solution also caused the absorption band at 670 mμ to disappear and produced the band of reduced choleglobin, but the reaction proceeded more slowly than the transformation of oxyhaemoglobin into reduced haemoglobin. Reoxygenation yields again the band at 670 mμ. In long-dialysed choleglobin solutions evacuation had no effect.

Another attempt was made starting from ferric choleglobin cyanide, the preparation of which is described below. Cyanide was removed by precipitation with ammonium sulphate from a solution reduced with $Na_2S_2O_4$ and by washing the precipitate with a saturated ammonium sulphate solution containing some phosphate buffer *p*H 7·6 and some $Na_2S_2O_4$. The precipitate was dissolved in water and the resulting solution reoxygenated. In this way the accompanying cyanmethaemoglobin was transformed into oxyhaemoglobin. There was an absorption band at about 680 mμ and CO failed to produce the band of CO-choleglobin without addition of $Na_2S_2O_4$. The band at 680 mμ differed, however, from that of the oxygenated choleglobin solution produced by ascorbic acid by not being removed by $Na_2S_2O_4$; it is, therefore, due to a ferrous haematin compound different from choleglobin, and not to oxygenated choleglobin. Again this throws no light on the nature of the compound responsible for the band at 670 mμ in oxygenated choleglobin solutions. Since we failed in our attempts to remove the last traces of reducing substances from choleglobin solutions without altering the oxygenated compound, we cannot decide whether this is metacholeglobin or oxycholeglobin; if oxycholeglobin exists, it must be more labile than oxyhaemoglobin.

Action of hydrogen peroxide on haemoglobin in the presence of cyanide

By the action of hydrogen peroxide on oxyhaemoglobin solutions containing potassium cyanide Barkan & Schales [1938] have obtained a green solution showing the following absorption spectrum after reduction with $Na_2S_2O_4$: I, 617–620, II, 564–568, III, 536–540 mμ. In repeating these experiments we found concentrations of 2% cyanide and 0·05–0·08% hydrogen peroxide optimal for the transformation into the green compound. The authors recognized that the absorption spectrum is due to the presence of two compounds, one causing the absorption band in the orange and the other the two bands in the green. The first substance they call 'pseudohaemoglobin' assuming that it consists of a bile pigment-iron complex bound to native globin. About the nature of the second substance they offer no definite suggestions, but apparently they believe that it is another product of oxidation of haemoglobin, no longer containing protohaem.

We will discuss this compound first, since the understanding of its nature will help us to understand that of the compound with the band at 617–620 mμ. The two bands in the green are those of ferrous denatured globin-cyan-protohaemochromogen. The bands can be produced without application of hydrogen peroxide by keeping an oxyhaemoglobin solution containing 2% cyanide 24 hr. at room temperature. Before reduction only one band at 542 mμ is seen which is that of cyancathaemoglobin (denatured globin-ferric protohaemochromogen cyanide). The denaturation of the globin is caused by the alkalinity of the cyanide. In the presence of hydrogen peroxide there is a more rapid oxidative denaturation, complete in 20 min. as shown by the change of the absorption spectrum of oxyhaemoglobin into that of cyancathaemoglobin. On careful neutralization or on dialysis against a neutral buffer complete precipitation occurs. Both the 'pseudohaemoglobin' and the compound with the bands in the green contain, therefore, denatured globin and not native globin.

Barkan & Schales have overlooked the identity of their second compound with ferrous cyanprotohaemochromogen probably because they observed a few spectroscopic properties, which hitherto have not been described for ferrous cyanprotohaemochromogen. In the weakly cyanide-alkaline solution and faster still in a neutral suspension or in a weakly acid solution the bands at 566 and

536 mμ of ferrous cyanprotohaemochromogen disappear and are replaced by a single rather diffuse band at 558 mμ, while in a more strongly alkaline solution they remain unaltered. NaOH added to the solution with the diffuse band at 558 mμ restores the original double-banded spectrum. As far as we know, the compound with the single band has so far not been described and its nature is unknown.[1]

Under conditions in which the single-banded compound arises from ferrous cyanprotohaemochromogen, CO transforms the latter at once into CO-protohaemochromogen (absorption bands at 570·5 and 540 mμ), while it does not react with it in strongly alkaline solutions. In a weakly cyanide-alkaline solution the position of the bands indicates an equilibrium between CO- and CN-compounds, which depends on the cyanide concentration and on the *p*H of the solution.

Under all these conditions the absorption bands in the green produced by the action of hydrogen peroxide behave exactly in the same manner as the bands of cyanprotohaemochromogen.

These observations in the protohaematin series allow us to interpret the nature of 'pseudohaemoglobin' and to explain the changes of its absorption studied by Barkan & Schales in a simple and more correct way. Dialysis, which removes the cyanide, transforms the compound into a substance with all the properties of cholehaemochromogen. 'Pseudohaemoglobin' differs from cholehaemochromogen in not reacting with CO in strongly alkaline solution reduced by $Na_2S_2O_4$; while coal gas shifts the band of cholehaemochromogen from 618 to 628 mμ, the band of 'pseudohaemoglobin' at 618 mμ remains unaltered. The compound is, therefore, a cyanide-compound, a conclusion also reached by Barkan. Evidently the substance is ferrous denatured globin cyancholehaemochromogen. Its behaviour towards CO is very similar to that of the corresponding protohaematin compound which accompanies it; it reacts with it only in acid or in weakly alkaline solution, in the latter somewhat less completely than the protohaematin compound.

Barkan & Schales believe that the dialysis of 'pseudohaemoglobin' causes an essential alteration changing 'pseudohaemoglobin' into 'pseudohaemochromogen'. If these names were taken at their face value, this would mean that the dialysis causes a denaturation of protein, which cannot be correct, the protein in 'pseudohaemoglobin' being already denatured. If we understand the authors correctly, they mean a slight alteration of the prosthetic group. Their evidence is based on their observations that the fresh cyanide-alkaline solution shows a shift of the absorption band with CO towards the infra-red while a dialysed solution to which the same amount of cyanide has been added, fails to show this shift. This is further complicated by another observation that if the order is reversed and the dialysed compound first transformed into the CO compound (band at 628 mμ), cyanide added now fails to shift the band back to 618 mμ. We cannot confirm these rather confusing observations. Provided that *p*H and cyanide concentration were actually restored, the dialysed solution to which cyanide had been added behaved exactly as the undialysed solution. Barkan's observation may be explained as follows: the fresh solution still contains some excess hydrogen peroxide and its reaction with $Na_2S_2O_4$ tends to acidify the

[1] Dicyanprotohaemochromogen, obtained by dissolving haemin in 10% KCN and adding $Na_2S_2O_4$ (absorption bands I, 572·4, II, 539·8 mμ, intensities II, I), can also be transformed into a similar single-banded compound with an absorption band at 555 mμ, if much $Na_2S_2O_4$ is added to a very dilute solution in water; the effect is due to the acidifying action of $Na_2S_2O_4$, alkali restoring again the two-banded spectrum.

solution, shifting the equilibrium between CO- and CN-compounds in favour of the former. We have failed to observe the final equilibrium to vary with the sequence in which the reagents were added, provided that sufficient time was allowed for the equilibrium to establish itself.

The globin denaturation during the action of hydrogen peroxide can be avoided by neutralizing the cyanide. When 0·05 % hydrogen peroxide acts on haemoglobin in the presence of 1 % KCN neutralized to litmus by KH_2PO_4, a compound with an absorption band in the red (at about 680 mμ) is formed which is soluble at *p*H 7·2. The band lies further towards the infra-red and is more diffuse than that of oxygenated choleglobin. Reduction with $Na_2S_2O_4$ produces reduced choleglobin. The compound does not react with CO without reducer, but yields CO-choleglobin after addition of $Na_2S_2O_4$. It is evidently cyanmeta-choleglobin (ferric choleglobin cyanide); a second absorption band in the green is that of cyanmethaemoglobin.

Attempts to isolate choleglobin from haemoglobin

Two different methods were tried, fractional precipitation with ammonium sulphate and differential adsorption on alumina. By the former no separation whatever was obtained. The ratio $\epsilon_{628} : \epsilon_{570}$ of the CO-haemochromogens was the same in the original solution, the filtrate and the solution of the precipitate.

When a solution with the ratio $\epsilon_{628} : \epsilon_{570}$ of the CO-haemochromogens = 0·37 was adsorbed on Willstätter's alumina C, the filtrate had a ratio of 0·36, the eluate from the alumina with 0·1 % ammonia a ratio of 0·405. The latter was brought to *p*H 6 by addition of acetate buffer *p*H 4·6 and again partially adsorbed on alumina. The ratio in the filtrate was this time 0·30 and in the eluate from the precipitate 0·50, but the eluate was slightly cloudy. Similar results were obtained with Brockmann's aluminium hydroxide (Merck) and with Fasertonerde according to Wislicenus (Merck). Choleglobin appears to be adsorbed preferentially, but the difference is too slight to encourage attempts at complete separation.

Summary

The preparation of choleglobin and cholehaemochromogen by the coupled oxidation of haemoglobin and ascorbic acid and the absorption spectra of these compounds are described. The oxidation of the haem part of the molecule, causing the formation of choleglobin, is soon followed by an oxidative denaturation of the globin part, leading to cholehaemochromogen.

Choleglobin and cholehaemochromogen reversibly combine with CO. Whether or not choleglobin combines reversibly with O_2, could not be established.

Cholehaematin is distinct from verdohaematin, although verdohaemochromogen is formed in the same type of reaction from pyridine-protohaemochromogen as is choleglobin from haemoglobin.

The 'pseudohaemoglobin' of Barkan & Schales, prepared by action of hydrogen peroxide and potassium cyanide on oxyhaemoglobin followed by reduction with sodium hydrosulphite, is ferrous denatured globin-cyan-cholehaemochromogen. With neutralized cyanide cyanmetacholeglobin is obtained. The solutions, however, contain by-products.

No effective way of separating choleglobin from haemoglobin was found.

Further oxidation of choleglobin and of cholehaemochromogen leads to compounds which in their absorption spectra resemble verdohaematin compounds, but which differ from them in some properties. Such products are also formed in the course of prolonged coupled oxidation of haemoglobin and ascorbic acid.

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34. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

3. QUANTITATIVE STUDIES ON CHOLEGLOBIN FORMATION. ESTIMATION OF HAEMOGLOBIN AND ASCORBIC ACID OXIDATIONS[1]

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In this paper we describe a spectrophotometric method allowing us to measure quantitatively the destruction of haemoglobin and the formation of choleglobin and cholehaemochromogen during the coupled oxidation of haemoglobin with ascorbic acid and with other reducing substances. The influence of various factors, the nature of the reactants, *p*H, temperature and oxygen pressure, on the velocity of the reaction is studied. The oxidation of ascorbic acid during the reactions is measured in the usual way by indicator titration, and the ratio of molecules of ascorbic acid oxidized to molecules of choleglobin formed is thus found. The spectrophotometric determination method enables us, also, quantitatively to correlate the formation of choleglobin with the increase of 'labile iron' and with the yield of bile pigments to be obtained after acid splitting, thus giving us a much more complete insight into the reaction; the results of these studies are described in Parts 4 and 5.

Experimental

Spectrophotometric determination of choleglobin formation

Technique. A large number of difficulties had to be overcome before a reliable technique could be developed. The solutions derived from the incubation of oxyhaemoglobin and ascorbic acid contain a mixture of oxyhaemoglobin, a little reduced haemoglobin, in some instances methaemoglobin, and reduced and oxygenated choleglobin; in the later stages of the reaction the precipitate of green pigment appears and even in the earlier stages a slight cloudiness occasionally develops. It is evident, therefore, that spectrophotometric study of the absorption of the solutions as such is useless. Means had to be devised to transform the various derivatives of protohaematin and of cholehaematin into one derivative of each and to ensure an optically clear solution. The transformation into the CO-haemochromogens by CO, NaOH and $Na_2S_2O_4$ fulfilled these demands. The CO-free haemochromogens are less suitable, being less stable. Even in the presence of CO we found it necessary to work under strictly controlled conditions in order to get reproducible results. Since $Na_2S_2O_4$ and O_2 yield peroxide which produces choleglobin, it is essential to add the reducer only after the solution has been saturated with coal gas and even then to avoid unnecessary exposure to air. We found it also necessary to add the alkali only after the solution had been saturated with coal gas. A CO-haemoglobin solution, which had an extinction coefficient at 570 mμ of 1·0, gave as CO-haemochromogen

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produced by addition of alkali and $Na_2S_2O_4$ after saturation with coal gas the extinction coefficients at 570 mμ: 1·00; 1·01; 0·98; 0·99. When alkali was added before the saturation with coal gas, the values were smaller and the variation greater: 0·81; 0·91; 0·80; 0·90; 0·91.

The following method was employed. 5 ml. of the solution to be investigated were at once saturated with coal gas for 3 min. The current of gas was as rapid as the frothing of the solution allowed. Only in a few instances did we find it necessary to add a trace of octyl alcohol, which tends to cause some denaturation. 50 mg. of $Na_2S_2O_4$ were then added, the tube once inverted and during further passage of coal gas 0·5 ml. of 20% NaOH was introduced. A vigorous current of coal gas was maintained for 1 min., after which the solution was transferred to the spectrophotometer screw cups. The extinction coefficients at 630 and 570 mμ were then measured without delay, although we found the absorption of the solutions in the cups to change only very slowly. The dilution to the concentration suited for spectrophotometric measurement must be carried out before the addition of $Na_2S_2O_4$ and alkali; the dilution by the alkali has, of course, to be considered. The instrument used was the Hilger Visible Spectrophotometer described in the preceding paper.

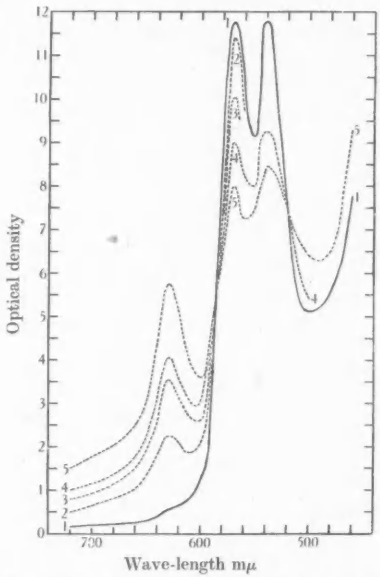


Fig. 1. Formation of choleglobin by coupled oxidation of haemoglobin and ascorbic acid. CO-haemochromogens. Curve 1: from haemoglobin. Curves 2, 3, 4 and 5: after 15, 30, 60 and 120 min. coupled oxidation at 37° respectively.

Changes of the absorption curves of the CO-haemochromogens during the incubation of oxyhaemoglobin and ascorbic acid

Fig. 1 shows the changes of the absorption curves of the CO-haemochromogens during incubation of a 1·2% oxyhaemoglobin solution with 100 mg. per 100 ml. of ascorbic acid at 37° and *p*H 8·5 in a culture flask without shaking (cf. Part 2).

The continuous line represents the curve of CO-protohaemochromogen, the interrupted lines those of the CO-haemochromogens obtained after varying times of incubation with ascorbic acid. The absorption of the CO-cholehaemochromogen at 630 mμ rises, while that of the CO-protohaemochromogen at 570 and 540 mμ decreases, the decrease of absorption at 540 mμ being somewhat less than at 570 mμ. Two isosbestic points are observed at 585 and 520 mμ. Owing to the steep course of the CO-protohaemochromogen curve at these wave-lengths the isosbestic point cannot be determined very accurately, but the curves display no evidence of the formation of more than one oxidation product during the duration of this experiment (120 min.).

Quantitative estimation of choleglobin

In order to determine the choleglobin concentration in a mixture with haemoglobin from the absorption of the CO-haemochromogens at 630 mμ it is necessary to know the specific extinction coefficients of CO-cholehaemochromogen at this wave-length. Pure CO-cholehaemochromogen has not yet been obtained; we can, however, calculate the specific extinction of CO-cholehaemochromogen from the measured ratio between the decrease of extinction at 570 mμ and the increase at 630 mμ if during the incubation only those compounds are formed which yield CO-cholehaemochromogen or CO-protohaemochromogen with CO, NaOH and $Na_2S_2O_4$. The data reported in the previous section indicate that this is the case, at least in the initial stages of the incubation. It is also necessary to know the ratios of the extinction coefficients at 630 and 570 mμ ($\epsilon_{630} : \epsilon_{570}$) of both CO-cholehaemochromogen and CO-protohaemochromogen. The latter ratio can be determined; the absorption of CO-protohaemochromogen at 630 mμ is small and the ratio $\epsilon_{630} : \epsilon_{570}$ for CO-protohaemochromogen (0·04 as average of a large number of determinations) has only the character of a correction factor. The ratio for CO-cholehaemochromogen cannot be determined correctly, but we arrived at the conclusion that it cannot be far from 2·22 (cf. p. 333, Part 2). If we use these values, the ratio ϵ_{630} of CO-cholehaemochromogen to ϵ_{570} of CO-protohaemochromogen, R, is obtained by the equation:

$$R = \frac{Q - 0{\cdot}04}{1 - Q/2{\cdot}22},$$

where Q is the observed ratio $\epsilon_{630} : \epsilon_{570}$. R ought to remain constant during the course of the reaction, if our assumption that cholehaematin derivatives are the only reaction products is correct. Table 1 shows that R is approximately 1·0 under widely varying conditions of the reaction. R does not remain absolutely constant during the reaction, but sufficiently so to indicate that at least during the early stages of the reaction choleglobin is practically the only product. Later, lower values of R are found, indicating some formation of by-products.

Table 1. *Values obtained for ratio (R) of ϵ_{630} of CO-cholehaemochromogen to ϵ_{570} of CO-protohaemochromogen from measurements of absorption decrease at 570 mμ and increase at 630 mμ*

*p*H 7·2, 37°		*p*H 8·5, 37°		*p*H 8·5, 20°	
Min. of incubation	R	Min. of incubation	R	Hr. of incubation	R
30	0·95	15	1·03	19½	0·96
40	0·91	30	1·08	31	0·84
80	0·83	40	0·96		
120	0·84	80	0·92		
		90	0·94		
		120	0·81		

For our calculations we assume $R=1$, i.e. equal maximal extinctions of CO-cholehaemochromogen and CO-protohaemochromogen. The specific extinction coefficient (extinction of a 0·1 % solution in 1 cm. layer) of CO-protohaemochromogen was found to be 0·965 at 570 mμ, and the same value is assumed for the specific extinction coefficient of CO-cholehaemochromogen at 630 mμ.

For the specific extinction coefficient at 630 mμ of the CO-cholehaemochromogen prepared from 'green pigment' we found the value 1·03 when the concentration was measured by the determination of the total iron in green pigment. A lower value (0·76) was obtained when the concentration was measured by weighing the protein denatured by formalin. Green pigment appears thus to contain some protein without strong absorption at 630 mμ and without iron. The correction for the protohaematin content in green pigment was based on the same data as the calculation of R from the ratio $\epsilon_{630} : \epsilon_{570}$, but the protohaematin content of green pigment is small and the correction therefore of much less importance. The agreement of the values obtained from green pigment with those obtained from choleglobin-haemoglobin solutions can, therefore, be taken as independent confirmation that the values arrived at for R and for the specific extinction coefficient at 630 mμ of CO-cholehaemochromogen are approximately correct.

We can now determine the content of cholehaematin and protohaematin derivatives in a solution. If ϵ°_{570} is the extinction coefficient at 570 mμ, of the haemoglobin solution before incubation with ascorbic acid, measured as CO-haemochromogen, and ϵ_{630} and ϵ_{570} the extinction coefficients at 630 mμ and 570 mμ after incubation, we have: choleglobin formed, in % of original haemoglobin $= \frac{102\,(\epsilon_{630} - 0{\cdot}04\,\epsilon_{570})}{\epsilon^{\circ}_{570}}$; and, unaltered haemoglobin $= \frac{102\,(\epsilon_{570} - 0{\cdot}45\,\epsilon_{630})}{\epsilon^{\circ}_{570}}$. If ϵ°_{570} is unknown, the choleglobin percentage of a mixture of choleglobin and haemoglobin is given by the equation: % choleglobin $= \frac{100\,(\epsilon_{630}/\epsilon_{570} - 0{\cdot}04)}{0{\cdot}96 + 0{\cdot}55\,\epsilon_{630}/\epsilon_{570}}$.

Estimation of protohaematin destruction

We have established the validity of the spectrophotometric method by estimating the destruction of protohaematin during the incubation of haemoglobin with ascorbic acid in a second way. By treatment of a haemoglobin solution containing ascorbic acid with acetic acid and ether, the protohaematin can be transferred almost quantitatively into the ether and can be determined spectrophotometrically as pyridine-protohaemochromogen in the ammoniacal extract. If this method is applied to choleglobin solutions, the ether contains beside protohaematin only biliverdin which is removed by extraction with 5 % HCl, and a small amount of biliviolin which does not disturb the haematin estimation.

For the determination the following technique was applied: 10 ml. of glacial acetic acid were added to 5 ml. of the choleglobin solution and the pigments were brought into 15 ml. of ether in the manner used for the determination of the bile pigments and described in detail in Part 5. The ether layer was repeatedly extracted with 5 % HCl, washed with water and finally the haematin was extracted with a few ml. of $N/10$ ammonia. Pyridine was added to a concentration of 20 % and after reduction with $Na_2S_2O_4$ the absorption at $\lambda = 557$ mμ (first absorption band of pyridine haemochromogen) was measured spectrophotometrically. The molar extinction coefficient of pyridine-protohaemochromogen at 557 mμ under these conditions was found to be 31,000.

We have made an interesting observation on the effect of ascorbic acid on the isolation of haematin from solutions of oxyhaemoglobin. 5 ml. of a 0·72% solution of haemoglobin ought to yield 1·405 mg. haemin. If 5 mg. of ascorbic acid were added to the haemoglobin solution immediately before the acidification with glacial acetic acid, 1·35 mg. of haemin = 96% were recovered from the ether. Without the addition of ascorbic acid 1·29 mg. = 92% were recovered, when the ether was added immediately after the addition of the glacial acetic acid; when, however, the acetic acid solution was kept for 15 min. before the addition of ether and the separation of the layers, the aqueous layer contained a great deal of haematin firmly bound to protein and no longer extractable by ether, and the yield of haemin isolated from the ether was only 0·51 mg. or 36% of the total haemoglobin-haemin. If acid haematin solution is kept in the presence of ascorbic acid, however, the yield of haematin does not diminish. Evidently ascorbic acid protects the globin in the acid solution from an alteration, probably of oxidative nature, which causes the protein-haematin linkage to become stable to acid.

Table 2. *Comparison of the spectrophotometric determination of choleglobin formation with the direct measurement of haemin destruction*

Conditions	mg. haemin from 5 ml. haemoglobin	% haematin destruction	% choleglobin according to spectrophotometric method
Initial haemoglobin	1·35	0	0
*p*H 7·2, 15 min.	1·215	10·0	6·9
30 „	1·19	11·9	14·1
*p*H 8·5, 15 min.	1·08	20·0	17·9
30 „	0·905	33·0	33·3

Table 2 shows that the percentage destruction of protohaematin estimated by this method tallies with the percentage formation of choleglobin as determined by the spectrophotometric method. The rather large deviations are due to the fact that the estimation of haematin destruction is less exact than the spectrophotometric estimation of choleglobin formation.

Measurement of the velocity of choleglobin formation under various conditions

Table 3 gives the results of measuring the reaction velocity of the choleglobin formation with this method under various conditions of temperature and *p*H. Each of the values given is the average of at least three parallel experiments. The haemoglobin concentration was 0·72%, that of ascorbic acid 100 mg. per 100 ml. and the incubation was carried out in flat culture flasks as previously described. As is to be expected from the nature of the experiment, variations are fairly large, but they do not affect the conclusions drawn from the experiments in any way. Table 4 shows the variation of a number of experiments carried out under the same conditions.

In the experiments in which a distinct precipitate of green pigment was found, a solution of the precipitate and the filtrate were analysed separately. The results of experiments of longer than 2 hr. can no longer be considered as reliable, since other compounds were formed in addition to choleglobin and cholehaemochromogen (cf. Part 2 and above). There is also occasionally bacterial contamination.

Table 3. *Choleglobin and cholehaemochromogen formation by coupled oxidation of haemoglobin with ascorbic acid*

		In % of initial haemoglobin					
Conditions	Duration of incubation	Chole-globin	Haemo-globin	Chole-haemo-chromogen	Proto-haemo-chromogen	Total chole-haem	Sum
*p*H 7·2, 37°	15 min.	6·9	92·7	0	0	6·9	99·6
	30 „	14·1	83·1	0	0	14·1	97·2
	80 „	21·6	55·1	13·0	0·8	34·6	90·5
	120 „	17·2	46·2	21·5	2·7	38·7	87·6
	7 hr.	22·4	28·2	34·3	3·7	56·7	88·6
	16 „	11·5	13·6	—	—	—	—
*p*H 8·5, 37°	15 min.	17·9	83·2	0	0	17·9	101·1
	30 „	33·3	69·7	Trace included in former		33·3	103·0
	80 „	27·9	49·7	17·6	1·5	45·5	97·6
	120 „	36·5	32·9	20·3	2·2	56·8	91·9
	7 hr.	20·0	26·5	21·4	6·2	41·4	74·1
	16 „	13·6	14·1	—	—	—	—
*p*H 7·2, 25°	30 min.	1·4	85·5	—	—	1·4	86·9
	60 „	6·1	83·8	—	—	6·1	89·9
	120 „	11·8	76·9	—	—	11·8	88·7
*p*H 8·5, 25°	30 min.	5·2	85·6	—	—	5·2	90·8
	60 „	16·5	82·1	—	—	16·5	98·6
	90 „	28·3	74·4	—	—	28·3	102·7
	120 „	42·3	56·1	Trace included in former		42·3	98·4

Table 4. *Variations in the yield of choleglobin and cholehaemochromogen*

30 min., *p*H 8·5, 37° In % of initial haemoglobin			80 min., *p*H 8·5, 37° In % of initial haemoglobin					
Chole-globin	Haemo-globin	Sum	Chole-globin	Haemo-globin	Chole-haemo-chromogen	Proto-haemo-chromogen	Total chole-haem	Sum
22·9	80·3	103·2	30·0	49·5	16·5	4·0	46·5	100·1
30·2	72·5	102·7	26·8	46·1	17·0	0·4	43·8	90·3
27·6	69·1	96·7	27·0	53·6	19·3	0·2	46·3	100·0
35·8	69·4	105·2	—	—	—	—	—	—
42·4	69·0	111·4	—	—	—	—	—	—
41·8	60·9	102·7	—	—	—	—	—	—
32·4	67·0	99·4	—	—	—	—	—	—
33·3 ± 2·7	69·7 ± 2·0	103·1	27·9 ± 1·0	49·7 ± 2·2	17·6 ± 0·8	1·5 ± 1·2	45·5 ± 0·9	96·7
			Standard deviation					
7·3	5·4	—	1·8	3·8	1·5	2·1	1·5	—

The reaction velocity of the choleglobin formation at *p*H 8·5 is initially more than double that at *p*H 7·2. The temperature coefficient is rather high (Q_{10} of the initial reaction about 3·5–4·0 between 25° and 37°). The concentration of choleglobin in the solution reaches a maximum, which is higher at 25° than at 37°; after this it remains constant, while more green pigment is formed. At first the latter contains little protohaemochromogen, but later the proportion of the latter increases. Finally the choleglobin concentration begins to decrease. Up to this point there is no indication of by-reactions, the sum of protohaematin and cholehaematin compounds being approximately 100 %. In the later stages, at *p*H 8·5 later than at *p*H 7·2, there is evidently destruction of choleglobin by further oxidation.

Formation of choleglobin by reducing substances other than ascorbic acid

Of other reducing substances reducton is of particular interest. Anderson & Hart [1934] describe the formation of green pigment by glucose + glycine + ammonia in alkaline phosphate buffers, and it is possible that reducton, chemically closely related to ascorbic acid, was the active agent in these mixtures. Crystalline reducton was prepared according to v. Euler & Martius [1933]. It is seen from the data in Table 5 that it undergoes coupled oxidation with haemoglobin

Table 5. *Formation of choleglobin by reducing substances other than ascorbic acid*

*p*H 7·2, 37°, haemoglobin 0·7 %, reducing substances in a concentration equivalent to 100 mg. per 100 ml. of ascorbic acid, in the case of thiolacetic acid to 50 mg. per 100 ml. of ascorbic acid.

		In % of initial haemoglobin					
Reducing substances	Incubation time	Chole-globin	Haemo-globin	Chole-haemo-chromogen	Proto-haemo-chromogen	Total chole-haem	Sum
Ascorbic acid	90 min.	25·4	61·9	18·3	0·3	43·7	105·9
Reducton (crystalline)	90 min.	33·8	54·6	8·4	4·6	42·2	101·4
Thiolacetic acid	90 min.	19·5	71·5	4·2	6·6	23·7	101·8
Glutathione, crystalline	19 hr.	24·4	65·6	5·6	4·8	30·0	Assumed 100
Glutathione, purified over Cd-salt	18½ hr.	6·5	—	Small amount insoluble in NaOH		—	—

and causes as rapid a formation of choleglobin as ascorbic acid. We were, however, unable to find reducton in the mixture used by Anderson & Hart by the indophenol reduction test. Thiolacetic acid also formed choleglobin only slightly less rapidly than did ascorbic acid. Glutathione caused only a very slow reaction, and even this seems to be brought about by impurities. There was considerably less choleglobin formed when glutathione purified over its cadmium salt was used. With cysteine no formation of choleglobin was observed, although a small amount of green precipitate was formed. Both glutathione and cysteine caused a considerable formation of methaemoglobin (cf. Table 6).

Formation of choleglobin in the presence of low concentrations of ascorbic acid

The experiments described hitherto were carried out with ascorbic acid concentrations of 50–100 mg. per 100 ml. In tissue cells the concentration of ascorbic acid is smaller, of the order of 5–50 mg. per 100 ml. (cf. Giroud [1937]). In guinea-pig spleen we found values of 6–15 mg. per 100 ml. with an average of 10·7 mg. per 100 ml.

We have, therefore, carried out some experiments with lower ascorbic acid concentrations. It must be borne in mind that with the comparatively large haemoglobin concentration required for the quantitative study of choleglobin formation, these small amounts of ascorbic acid are rapidly destroyed. In the tissues, however, fresh ascorbic acid can be made available by back-reduction of dehydroascorbic acid with reduced glutathione [Borsook *et al.* 1937; Schultze *et al.* 1937–38; cf. also Genevois & Cayrol, 1939]. Lemberg *et al.* [1938, 1] have shown that reduced glutathione saves ascorbic acid from the oxidation by haemochromogen and does not prevent the oxidation of haemochromogen to verdohaemochromogen. Reduced glutathione accompanies ascorbic acid in the cells of liver and spleen in rather high concentration. In guinea-pig spleen we found 207 mg. reduced glutathione per 100 g. of fresh organ. We have therefore

studied the action of small concentrations of ascorbic acid on oxyhaemoglobin in the absence and in the presence of glutathione on the formation of choleglobin and the results are given in Table 6.

Table 6. *Formation of choleglobin with low concentrations of ascorbic acid in the presence of glutathione*

0·14 g. of haemoglobin in 20 ml. phosphate buffer *p*H 7·2, 37°

Ascorbic acid mg.	GSH mg.	ϵ_{630} of CO-haemochromogens after 90 min. incubation	Biliverdin after acid splitting of 5 ml.	Spectroscopic observations after 2½ hr. incubation
—	—	0·27	None	Oxyhaemoglobin
—	50	0·39	Trace	Oxyhaemoglobin and methaemoglobin; band 634 mμ disappearing on reduction with $Na_2S_2O_4$
2	—	0·62	Trace	Band at 630 mμ resistant to $Na_2S_2O_4$
2	50	1·05	Considerable amount	Band at 630 mμ resistant to $Na_2S_2O_4$; some green pigment
20	—	2·07	Large amount	Band at 630 mμ resistant to $Na_2S_2O_4$; large amount of green pigment

Even small concentrations (10 mg. per 100 ml.) of ascorbic acid cause a noticeable formation of choleglobin, much larger than that produced by glutathione in a concentration of 250 mg. per 100 ml. In the presence of both glutathione and ascorbic acid more choleglobin is formed than by ascorbic acid alone, and the increase in absorption is significantly larger than can be explained by additive effect. Under the conditions prevailing in the tissue cell haemoglobin must, therefore, undergo oxidation to choleglobin. Actually the condition in the tissue cells is even more favourable than in our experiment, as it will be seen below that low oxygen pressure increases the rate of choleglobin formation while not affecting the destruction of ascorbic acid.

Rate of oxidation of ascorbic acid and effect of oxygen partial pressure on the oxidation of haemoglobin and ascorbic acid

Table 7 gives the percentage losses of ascorbic acid during the incubation of ascorbic acid (100 mg. per 100 ml.) with a 0·7 % haemoglobin solution at 37° and *p*H 7·2 and 8·5; the reaction mixture was kept in flat culture flasks under the conditions previously described.

Table 7. *Ascorbic acid destruction*

Incubation time in min.	% loss of ascorbic acid	
	*p*H 7·2	*p*H 8·5
30	10	26
45	—	—
60	23	—
90	31	45
120	40	—
155	—	55
180	51	—
225	—	74

By comparison with the rate of choleglobin formation given in Table 3 the oxidation ratio—molecules of ascorbic acid oxidized per molecules of haemo-

globin oxidized to choleglobin—can be found for the time experiments. This ratio is 10·6 after 30 min. and 11·7 after 80 min. incubation at *p*H 8·5; at *p*H 7·2 the values are 9·6 after 30 min. and 14·0 after 120 min. If 1 mol. O_2 is required to oxidize haemoglobin to choleglobin and 0·5 mol. to oxidize ascorbic acid to dehydroascorbic acid, about one-sixth of the O_2 taken up during the first 30 min. at either *p*H is used for the formation of choleglobin and five-sixths for that of ascorbic acid. Actually somewhat more O_2 will be used for the further oxidation of dehydroascorbic acid.

In order to study the effect of the O_2 partial pressure on the oxidation of haemoglobin and ascorbic acid and on the oxidation ratio under more defined conditions we have carried out experiments in Warburg flasks in which the reaction mixture was shaken with air and with a nitrogen-air mixture containing 2% O_2. We have shown [Lemberg *et al.* 1938, 2] that in the coupled oxidation of pyridine haemochromogen and ascorbic acid lowering of the O_2 concentration decreases the oxidation ratio from 59 in O_2 to 32 in air and to 12 in a nitrogen-air mixture containing 4% O_2. The rate of oxidation of pyridine-haemochromogen was little affected, while that of the ascorbic acid oxidation was proportional to the O_2 partial pressure. Table 8 shows that in the coupled

Table 8. *Effect of O_2 pressure on choleglobin formation and ascorbic acid destruction*

Haemoglobin 0·7%; ascorbic acid 100 mg. per 100 ml.; *p*H 7·2; 37°.
Total volume of solution: 2·6 ml.; in central cup, 0·4 ml. KOH.

O_2 concentration	Piperidine dithiocarbamate	Incubation time min.	O_2 consumption μmol.	Ascorbic acid oxidation μmol.	Choleglobin in % of original haemoglobin	Formation in μmol.	Oxidation ratio	(mol. O_2 consumed)/(mol. choleglobin formed)
20%	Absent	30	2·41	4·06	4·8	0·05	81	48
		45	2·90	4·92	9·9	0·10	49	29
Standing in air	Absent	30	—	1·48	14·1	0·155	10	—
2%	Absent	30	2·34	4·52	—	—	—	—
		45	3·11	5·80	38·1	0·38	15	8·5
20%	100 μg.	30	4·7	—	—	—	—	—
		45	6·5	—	23·8	0·23	< 54	28
2%	100 μg.	30	3·9	—	—	—	—	—
		45	5·1	—	60	0·58	~ 16	9

oxidation of haemoglobin and ascorbic acid the oxidation ratio is also much lower with low O_2 concentration. Here, however, the rate of oxidation of ascorbic acid is little affected, while that of the choleglobin formation is greatly increased with low O_2 concentrations.

Whatever may be the correct explanation of the reaction mechanism (cf. below), it is clear that the low O_2 pressure in the tissue cells favours the oxidation of haemoglobin to choleglobin.

The rate of choleglobin formation in laked erythrocytes

For the experiments described hitherto, solutions of crystalline horse oxyhaemoglobin were used. Table 9 gives the measurements of the velocity of choleglobin formation by coupled oxidation with ascorbic acid under the same conditions as those of Table 3, using, however, laked horse erythrocytes diluted to a haemoglobin concentration of 0·72%, instead of the solution of oxyhaemoglobin.

Table 9. *Choleglobin formation in laked cells with ascorbic acid*

Conditions	Incubation time in min.	Choleglobin in % of initial haemoglobin	
		Laked cells	Laked destromatized cells
*p*H 7·2, 37°	15	1·3	5·3
	30	5·1	15·0
*p*H 8·5, 37°	15	22	—
	30	35	—

A comparison with the data compiled in Table 3 shows that at *p*H 8·5 the reaction velocity is almost the same, when laked horse cells are used instead of a solution of horse oxyhaemoglobin. At *p*H 7·2, however, choleglobin is formed much more slowly from laked red cells. This indicates the presence in the erythrocytes of a factor inhibiting the formation of choleglobin. This factor is contained in the stromata. When the stromata are removed by keeping the laked cells at a *p*H of 5·8 in the refrigerator and centrifuging, the haemoglobin of the destromatized red cells is now attacked by ascorbic acid and O_2 as rapidly as that in a solution of crystalline oxyhaemoglobin.

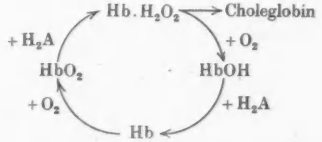
These observations help us to explain why there is no, or at least only a very slow, formation of choleglobin in the red cells. From the figures given in Table 6 it can be calculated that, in the presence of 250 mg. per 100 ml. of glutathione, ascorbic acid in a concentration of 10 mg. per 100 ml. is still able to transform 12% of 0·7% haemoglobin into choleglobin in 90 min. The concentration of ascorbic acid in the erythrocytes is still considerably smaller. In rabbit erythrocytes we found only 0·35 mg. per 100 ml., considerably less than the values reported by Stephens & Hawley [1936] for human erythrocytes (0·85–1·2 mg. per 100 ml.); in rabbit's plasma are found higher concentrations than in the cells (0·8–1·9 mg. per 100 ml.), while Stephens & Hawley report 1·1–1·35 mg. per 100 ml. for normal human plasma. From our figures it can be concluded that the membrane of the red cell offers some protection to the haemoglobin contained in it by its very incomplete permeability to ascorbic acid [cf. also Borsook *et al.* 1937]. Even so one would still expect a slow, but substantial haemoglobin destruction in the erythrocytes. This is further decreased, if not prevented, by the inhibiting factor in the stroma, which in the cell is probably still much more effective than in the great dilution in which it is present in our dilute laked cell solutions. Whether these factors are able to prevent the formation of choleglobin or other bile pigment-haemoglobins in the red cell completely, is doubtful, and this problem will be discussed in the succeeding paper.

The protection of the plasma ascorbic acid from oxidation by the red cells [Kassan & Roe, 1940] is probably due to this stroma factor rather than to the glutathione of the red cell, with which the plasma ascorbic acid is not in direct contact; Borsook *et al.* [1937] did not find evidence for the reduction of dehydroascorbic acid added to blood. The rapid destruction of ascorbic acid in the blood following haemolysis is due to the coupled oxidation with haemoglobin.

The reaction mechanism

In Part I of this series [Lemberg *et al.* 1939] we have shown that the formation of choleglobin by the coupled oxidation of haemoglobin with ascorbic acid cannot be due to hydrogen peroxide formed by autoxidation of ascorbic acid, since the reaction takes place in the presence of copper inhibitors which prevent the

autoxidation. Nevertheless, hydrogen peroxide acting on haemoglobin in the presence of ascorbic acid forms choleglobin. We have explained these facts by assuming as first step of the reaction hydrogen transfer from ascorbic acid to oxyhaemoglobin with the formation of a ferrous haemoglobin-H_2O_2 compound, and have assumed the following cycle in order to explain the destruction of many more ascorbic acid molecules than haemoglobin molecules in the coupled oxidation:



Hb reduced haemoglobin
HbO_2 oxyhaemoglobin
HbOH methaemoglobin
H_2A ascorbic acid

We assume the ferrous haemoglobin-hydrogen peroxide compound to undergo an intramolecular peroxidative reaction, the porphyrin ring being oxidized by the O_2 of the iron-bound hydrogen peroxide of the same molecule.

The reaction mechanism of the formation of verdohaemochromogen from pyridine-haemochromogen by coupled oxidation with ascorbic acid was explained in a similar way [Lemberg *et al.* 1938, 1, 2]. We have found that hydrogen peroxide acting on pyridine-haemochromogen in the presence of ascorbic acid and in the absence of O_2 leads to a precursor of verdohaemochromogen [Lemberg *et al.* 1938, 2]. The green compound obtained by Haurowitz [1937] by reduction of the ferric pyridine haemochromogen-hydrogen peroxide compound by pyrogallol is also verdohaemochromogen.

Hydrogen peroxide forms choleglobin from haemoglobin in the presence of ascorbic acid. We have now found that even in the absence of reducing substances hydrogen peroxide causes a substantial formation of choleglobin from reduced haemoglobin, while much less is formed from oxyhaemoglobin. To 3·5 ml. of an 0·7 % haemoglobin solution, reduced by evacuation under nitrogen, 0·5 ml. of 0·6 % hydrogen peroxide was added under nitrogen. After 2 min. at 20° the excess hydrogen peroxide was destroyed by a drop of catalase solution and the choleglobin content determined spectrophotometrically. It was found that 16 % of the reduced haemoglobin had become transformed into choleglobin. If oxyhaemoglobin reacted with hydrogen peroxide under the same conditions but in air, only 7 % was transformed into choleglobin. Methaemoglobin yielded 6 % choleglobin under nitrogen. Hydrogen peroxide is evidently able to reduce some methaemoglobin to haemoglobin in the same way as catalase is reduced to its ferrous form by hydrogen peroxide [Keilin & Hartree, 1937].

These observations show that the effect of the O_2 pressure on the oxidation ratio (cf. above and Table 8) can be understood as autoxidation of the ferrous haemoglobin-H_2O_2 compound competing with its transformation to choleglobin. The methaemoglobin-hydrogen peroxide compound is known to be unstable and to give methaemoglobin. The fact that high O_2 pressure lowers the oxidation ratio cannot be explained by the assumption that the autoxidation of ascorbic acid competes with its coupled oxidation with haemoglobin, since even at high O_2 pressure copper inhibitor does not diminish the oxidation ratio and the effect of O_2 pressure in the oxidation ratio in the presence of copper inhibitor is the same as in its absence.

We have ascertained that methaemoglobin can be an intermediate product in the cycle. It reacts with about the same velocity as oxyhaemoglobin, and the effect of O_2 pressure on the ratio 'mol. of O_2 consumed to mol. of

Table 10. *Choleglobin formation from methaemoglobin*

Methaemoglobin 0·7 %; ascorbic acid 100 mg. per 100 ml.; *p*H 7·2; 37°. Volume of solution: 2·6 ml.; in central cup, 0·4 ml. KOH.

O_2 concentration	Incubation time min.	O_2 uptake in μmol.	Choleglobin formation: In % of original haemoglobin	Choleglobin formation: In μmol.	Ratio (mol. O_2 consumed)/(mol. choleglobin formed)
20 %	1	0·36	—	—	—
	15	1·69	—	—	—
	30	2·59	—	—	—
	45	3·35	8·4	0·094	36
2 %	1	0·13	—	—	—
	15	1·20	—	—	—
	30	2·00	—	—	—
	45	2·90	24·6	0·276	10·5

choleglobin formed' is the same as with oxyhaemoglobin (compare Table 10 with Table 8).

While the effects of the O_2 pressure on the oxidation ratios in choleglobin and verdohaemochromogen formation and on the reaction velocities of verdohaemochromogen formation and destruction of ascorbic acid in it can be understood by the notion that the autoxidizable ferrous haem compounds undergo oxidation of their prosthetic groups, it still remains unexplained why the optimum of the rate of choleglobin formation is at low O_2 pressure (cf. Table 8), while the rate of verdohaemochromogen formation does not depend on O_2. Since the effects of the O_2 pressure on the oxidation of ascorbic acid also differ in both cases in an analogous manner (a high O_2 pressure favouring the destruction of ascorbic acid in the coupled oxidation with pyridine-haemochromogen, but no influence of O_2 pressure on ascorbic acid oxidation with haemoglobin), it is likely that an explanation of the phenomenon must be concerned with the reaction initiating the cycle, the reaction of oxyhaemoglobin with ascorbic acid. There is a remarkable parallel between choleglobin and methaemoglobin formations from oxyhaemoglobin, the rates of both reactions having maxima at low O_2 pressures in spite of the fact that the reactions require O_2. The range in which this optimum is found, is one in which there is partial dissociation of oxyhaemoglobin. The observations of Brooks [1935] make it likely that the explanation has to be sought in an intermediate between Hb_4 and Hb_4O_8, e.g. Hb_4O_2, being the substance converted into methaemoglobin. The same applies probably to choleglobin formation. Brooks himself rejects this explanation on the basis of calculations involving various constants which hitherto have not been established securely. His own explanation is, however, quite unsatisfactory. Besides assuming an inhibition by O_2 of obscure nature, he believes that the rate of methaemoglobin formation is proportional to the concentration of reduced haemoglobin which is decreased by high O_2 pressure. Brooks overlooks the fact that the concentration of reduced haemoglobin is low at high O_2 pressure, because it reacts more rapidly with O_2. There is no reason to assume that this reaction should lead to oxyhaemoglobin exclusively, while another reaction of reduced haemoglobin with O_2 would yield methaemoglobin.

Discussion

In a previous paper [Lemberg *et al.* 1938, 1] we had put forward the hypothesis that dehydroascorbic acid, formed by the coupled oxidation of haemoglobin and ascorbic acid during the bile pigment formation *in vivo*, is reduced

back to ascorbic acid by reduced glutathione, and that oxidized glutathione in turn is reduced by the glucose dehydrogenase system. The latter assumption was based on the experiments of Mann [1932]. Meanwhile Schultze *et al.* [1939] have shown that neither ascorbic acid nor glutathione nor a mixture of the two substances can act as a hydrogen-carrying system between nicotine-haemochromogen and the glucose dehydrogenase system or coenzyme. This result scarcely affects our hypothesis that the ascorbic acid-glutathione system plays an important part in the physiological destruction of haemoglobin, since it is not of importance how the glutathione is restored. What principally matters is that ascorbic acid and haemoglobin undergo coupled oxidation under the conditions found in the cells of those organs in which bile pigment formation takes place; for this we bring forward evidence in the present paper. Secondly, ascorbic acid must be somehow restored, because the daily haemoglobin destruction involves the oxidation of a larger amount of ascorbic acid than is replaced in the food. The fact that dehydroascorbic acid has antiscorbutic activity makes it almost certain that it can be reduced *in vivo*. Several authors (cf. above) have shown that reduced glutathione is able to reduce dehydroascorbic acid in liver cells as well as *in vivo*, but recently Schultze *et al.* [1937–38] have thrown some doubts on the actual occurrence of this reaction *in vivo*. We agree with those authors that their results make it unlikely that the ascorbic acid-glutathione system plays a major role in cell respiration [cf. King, 1939]. A sweeping generalization that the reduction of dehydroascorbic acid by glutathione does not occur in the animal body would, however, not be justified. The experiments of Schultze *et al.* [1937–38, p. 403] were carried out with a proportion of glutathione to ascorbic acid which was much lower than that actually found in liver and spleen, and even so they showed only that the reduction of dehydroascorbic acid by glutathione was not complete.

Other reducing systems may play a role in the physiological oxidation of haemoglobin, but so far the ascorbic acid-glutathione system is the only one known to occur in cells that is able to catalyse the formation of choleglobin under physiological conditions.

Summary

A spectrophotometric method has been worked out for the determination of the choleglobin content in mixtures of choleglobin and haemoglobin, and thus the measurement of the rate of choleglobin formation.

The spectrophotometric investigation indicates that in the initial stages of the reaction choleglobin is the only product of oxidation of haemoglobin, while later other products are formed in addition to choleglobin and cholehaemochromogen.

The initial reaction velocity of choleglobin formation is about twice as large at *p*H 8·5 as it is at *p*H 7·2. The temperature coefficient is high ($Q_{10}=3{\cdot}5-4$ between 25 and 37°).

Reducton forms choleglobin as fast as ascorbic acid, thiolacetic acid more slowly, glutathione and cysteine very slowly, if at all.

Choleglobin is rapidly formed from haemoglobin under physiological conditions (*p*H 7·2, 37°) and with concentrations of glutathione and ascorbic acid such as occur in tissue cells, glutathione increasing the rate of choleglobin formation more than can be due to a merely additive effect.

At low O_2 pressure (15 mm.) the formation of choleglobin proceeds faster than in air, while the oxidation of ascorbic acid is little affected. The same is found in experiments carried out in the presence of copper inhibitor which prevents the

autoxidation of ascorbic acid. The theoretical implications of these observations are discussed. In air, without shaking, about 10 molecules of ascorbic acid are oxidized per molecule of choleglobin formed.

In the red cells haemoglobin is protected from rapid oxidation by the small permeability of the cell membrane to ascorbic acid and by an inhibiting factor in the stromata.

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35. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

4. THE 'LABILE IRON' IN BLOOD AND ITS INCREASE DURING CHOLEGLOBIN FORMATION[1]

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It has been shown by Lemberg [1935] and Lemberg & Wyndham [1937] that the iron of a bile pigment-haematin compound (verdohaemochromogen) is more easily detached by acids than haemoglobin iron. If choleglobin is a bile pigment-haemoglobin it is to be expected that its formation during the coupled oxidation of haemoglobin and ascorbic acid will be accompanied by an increase in the 'labile iron'.

Barkan and his collaborators have shown in several papers that a small part of the iron in erythrocytes is split off more readily by acids than that of the remainder, and that 'leicht abspaltbares Bluteisen', for which we shall write in short ESI, accompanies oxyhaemoglobin through its purification and recrystallization. The initial values of ESI have, therefore, to be taken into account in the study of ESI in choleglobin solutions. According to Barkan & Schales [1937; 1938] the source of ESI is a bile pigment-haemoglobin ('pseudohaemoglobin') accompanying haemoglobin in the red cells, and inseparable from it on account of the identity of the protein parts (native globin). If this be correct, pseudohaemoglobin and choleglobin must be identical or very closely related. We have therefore reinvestigated the problem of the ESI in erythrocytes. While our experimental results agree with those of Barkan, except in one or two points, we arrive at different conclusions as to the explanation of these observations.

By this investigation we have also established the values of the initial ESI obtained from haemoglobin solutions in the presence of ascorbic acid in such concentrations as were used for the coupled oxidation. Thus we are enabled to measure the increase of ESI occurring during this reaction and to correlate it with the increase of choleglobin concentration, estimated spectrophotometrically with the method described in the preceding paper.

Experimental

Methods

Determination of ESI. Barkan incubates the diluted solution of haemoglobin with 0·4 % HCl for 16–24 hr., and then obtains by ultrafiltration a protein-free solution, in which he estimates the iron by a ferric thiocyanate method.

We found that our ultrafiltrates through Zsigmondy filters ('Cella finest' and 'Ultrafine') contained some protein breakdown products. These came out of solution at the *p*H used for the colorimetric estimation of iron as the ferrous $\alpha\alpha'$-dipyridyl complex in the method of Hill after Shorland & Wall [1936]. In

[1] The work was carried out under a grant from the National Health and Medical Research Council of Australia.

order to use this method we prepared protein-free filtrates by trichloroacetic precipitation after incubation with HCl; blanks were, of course, carried out and the blank values subtracted. The results did not differ from those obtained with the Barkan method. A choleglobin solution, which according to the latter contained 10·25 % and in the presence of CO 11·3 % ESI, gave the values 11·0 and 10·25 % with our method. Barkan [1937] has also made use of trichloroacetic acid precipitation and confirmed earlier results of other workers that it gives the same results as the rather tedious ultrafiltration. He claims that in the presence of CO too low values of ESI in blood are occasionally found with the trichloroacetic acid method, so that the CO inhibition of the removal of ESI appears too high. The CO inhibitions that we observed in blood were, however, the same as those found by Barkan.

As a routine method we adopted the following procedure. To 1 ml. of the solution to be determined (blood diluted 10 times or choleglobin solution prepared from 0·7 % haemoglobin undiluted) was added 1 ml. of iron-free 0·8 % HCl; after mixing the solution was incubated overnight (16 hr.) at 37°. 1 ml. of 7 % trichloroacetic acid was then added, the mixture well shaken and the precipitate filtered off on a Whatman filter No. 42. The iron in an aliquot of the filtrate was then estimated by the method of Shorland & Wall.

If the acid treatment had to be carried out in CO or N_2, the solution was placed in the bottom part of a Thunberg tube and the HCl in the bent stopper. The CO was kept standing over alkaline pyrogallol solution, while the N_2 was freed from O_2 over heated copper asbestos.

Determination of total iron. A suitable aliquot of the solution was taken to dryness in a 6 in. × $\frac{5}{8}$ in. pyrex test tube. After ashing with 0·4 ml. conc. H_2SO_4 and 0·1 ml. perchloric acid (Merck) the solution was cooled and either made up to 10 ml. with glass-distilled water and analysed by the dipyridyl method; or after 2–3-fold dilution with water the solution was made alkaline with 5*N* NH_4OH containing ammonium thiolacetate. During this procedure it was cooled with ice water. After keeping for 5–10 min. with repeated shaking in order to keep the complex in the ferric state, the solution was compared colorimetrically with an iron standard treated in the same way. The two methods gave identical results.

The blood ESI and its behaviour towards CO and reducing substances

Thirteen determinations of the ESI on seven samples of human blood exposed to air (ESI_{O_2}) gave results which varied from 3·6 to 7 % of the total iron, the mean being 5·35 % and the standard deviation ± 1·17 %. Thirteen determinations on the same batch of crystalline horse oxyhaemoglobin gave values ranging from 3·5 to 11 %, with a mean of 6·9 % and a standard deviation of ± 2·5 %. During the preparation of crystalline horse oxyhaemoglobin the ESI_{O_2} varied from 4·9 % in the cells to 5·4, 5·7 % in the first crystallization, 4·0, 3·5 % in the second and 8·5, 9·8 % in the third. Recrystallization thus did not alter the ESI_{O_2} significantly. These values agree with those found by Barkan and other observers.

Effect of carbon monoxide. We can also confirm that under CO much less iron is detached than in air. The ESI observed under CO (ESI_{CO}) is only about one-third of that observed when the splitting is carried out in air (ESI_{O_2}). The average amounts of ESI_{CO} as % of ESI_{O_2} in thirteen estimations on crystalline horse oxyhaemoglobin varied from 21 to 47 with a mean of 31 and a standard deviation of ± 5. Determinations on sheep blood came within this range. This would mean an inhibition of the detachment of iron by CO of 69 % on the average.

Effect of reducing substances and inert gases. Barkan & Berger [1928] have already found that H_2 has an effect similar to that of CO.

Table 1 shows the effect of evacuation and addition of reducing substances ($Na_2S_2O_4$ and ascorbic acid) on the detachment of iron. These experiments were carried out with both crystalline horse oxyhaemoglobin and laked sheep cells. No difference was observed, so they are given together. Evacuation and ascorbic acid in concentrations of 30 mg. per 100 ml. or more decrease the detachment of iron by acids to the same degree as CO or H_2. The amount split off in presence of $Na_2S_2O_4$ is a little larger, because the SO_2 developed on acidification of the solutions removes some iron from haemoglobin.

Table 1. *Influence of evacuation and reducing agents on blood ESI*

Conditions	ESI found in % of ESI_{O_2}	No. of experiments averaged
In vacuo	33	2
Added $Na_2S_2O_4$, 50 μg. per ml.	89	2
„ 100 „	51	5
„ 300 „	46	8
„ excess	53	4
Added ascorbic acid, 50 μg. per ml.	80	2
„ 100 „	80	6
„ 300 „	38	6
„ 1200 „	25	2

Barkan & Schales [1937] claim that by the addition of small amounts of $Na_2S_2O_4$ the inhibition of the iron detachment by CO can be further increased, and base important conclusions on this observation. While the theoretical side of the question will be discussed later, we point out here that the experiments of Barkan & Schales lend only slender support to this claim. The authors report three experiments in which the ESI_{CO} was further lowered by addition of 130–330 μg. $Na_2S_2O_4$ per ml. Examination of their figures reveals, however, that in two of these experiments the amount split under CO in the absence of $Na_2S_2O_4$ was abnormally high (ESI_{CO} still 75 % and 58 % of ESI_{O_2}, instead of the usual 31 %); and that ESI_{CO} in the presence of the reducer (25 % of ESI_{O_2}) was not significantly smaller than usually without reducer and still inside the range of the figures for ESI_{CO} in percentage of ESI_{O_2} (21–47 % in our experiments). It is very likely that the saturation of these solutions with CO was somehow incomplete and that the effect of the reducer was only to bring it to completion. In one experiment only ESI_{CO} was 33 % of ESI_{O_2} without reducer and was further decreased by the addition of $Na_2S_2O_4$ to 14 %. Occasional stray results do, however, occur and cannot be considered as sufficient evidence.

We have therefore repeated these experiments using various concentrations of $Na_2S_2O_4$ and also of ascorbic acid added to solutions of crystalline horse oxyhaemoglobin (Table 2).

Table 2. *Influence of reducing agents on blood ESI in the presence of CO*

Conditions	ESI_{CO} in % of ESI_{O_2}
Without reducer, CO	28
Added $Na_2S_2O_4$, 130 μg. per ml.	14; 31
„ 300 „	31
Added ascorbic acid, 50 μg. per ml.	32
„ 120 „	15; 29
„ 300 „	28
„ 1200 „	24

Again a decrease of the iron split off was only observed in two experiments out of eight, and in each case repetition of the same experiment failed to confirm the low value. The decrease cannot therefore be considered as significant.

Experiments on the nature of the compound from which the blood ESI is derived

In the first paper of this series [Lemberg, Legge & Lockwood, 1939] we have already mentioned that CO does not decrease the amount of ESI liberated from choleglobin solutions, and have adduced this as evidence against the identity of choleglobin and the substance in blood which is the source of the ESI. The choleglobin solutions contain, however, ascorbic acid in concentrations between 50 and 100 mg. per 100 ml. and from Tables 1 and 2 it is evident that the blood ESI would also not be decreased much further by CO in the presence of ascorbic acid in this concentration. After removal of ascorbic acid from choleglobin solutions by prolonged dialysis CO has still no effect on the yield of ESI, but during dialysis oxycholeglobin, if present, may be transformed into ferric choleglobin and therefore not react with CO. It cannot be absolutely excluded, therefore, that CO may inhibit the removal of iron from an oxycholeglobin with reversibly bound O_2, the existence of which is not proved. It will be seen from the discussion below, that this point is no longer of importance, since we believe that the CO inhibition of the detachment of ESI in the blood has to be explained in a totally different way.

We have also briefly described spectroscopic observations which made us doubt the occurrence of choleglobin in red cells. They are given here in greater detail. When a strong solution of oxyhaemoglobin or of laked erythrocytes is treated with $Na_2S_2O_4$ in presence of air, the band of choleglobin is observed, and that of cholehaemochromogen when the haemochromogen is formed by pyridine or alkali and $Na_2S_2O_4$ without precautions. When, however, reduced haemoglobin is formed by evacuation or when the reductions with $Na_2S_2O_4$ are carried out in a medium free from O_2, no trace of these bands can be observed spectroscopically. In the first-mentioned experiments choleglobin is, therefore, an artefact, doubtless formed from haemoglobin by the action of traces of hydrogen peroxide, which is well known to arise by the action of O_2 on $Na_2S_2O_4$ solutions. While these experiments show once more the ease with which choleglobin arises from haemoglobin, they do not offer any evidence for its occurrence in red cells. In the absence of O_2 another absorption band was observed in solutions of reduced haemoglobin and in denatured globin and pyridine haemochromogens, lying at about 660 mμ. This band disappeared on oxygenation and reappeared on reduction. It was not observed in solutions of CO-haemoglobin or in laked blood treated with coal gas with or without addition of $Na_2S_2O_4$. This compound may be a source of some of the blood ESI.

From the spectrophotometric curves of CO-haemochromogen prepared from laked blood or crystalline oxyhaemoglobin (Fig. 1 of Part 3) we can deduce that choleglobin iron cannot form as much as 5 % of the total iron. There is only a very slight hump of the absorption curve at 630 mμ indicating less than 1 % of choleglobin. Even if pure CO-protohaemochromogen had zero absorption at 630 mμ, which from the form of the absorption curve is very unlikely, the choleglobin content could not be higher than 3·9 %.

We have already briefly reported that the treatment of laked erythrocytes and of horse oxyhaemoglobin with acid yields, indeed, some bile pigment, and quantitative estimations will be given in the succeeding paper. While this proves the occurrence of some bile pigment haemoglobin in red cells, the bile pigment

yield was much lower than that expected from a bile pigment haemoglobin content of 5% which ought to be present if all ESI were derived from it.

We have therefore investigated whether some of the ESI may not originate from haemoglobin. The arguments which Barkan brings forward against this assumption will be discussed below, while here we present a few experiments. We have tried whether methaemoglobin synthesized in a way which excludes the presence of bile pigment-iron compounds, does not yield any ESI. Pure crystalline haemin should not, according to the way it is prepared, contain any bile pigment haematin; we have further made sure of this by extracting its ether-pyridine solution repeatedly with 20% HCl. After washing with water the precipitated haemin was dissolved in $N/10$ ammonia and coupled at once with the optimal amount of a native horse globin solution, the iron content of which was known. The alkaline haematin solution must not be allowed to stand, since we have observed that it is not very stable and after a few hours a band may be found at about 630 mμ after addition of native globin and $Na_2S_2O_4$. The synthesized methaemoglobin yielded even slightly more ESI (6·5–9·6%) than oxyhaemoglobin from red cells (Table 3). Inhibition by CO was not found, nor was it expected since the iron is in the ferric state. The experiment shows that the ESI of the blood may, at least partly, be derived from haemoglobin.

Table 3. *ESI in synthesized methaemoglobin*

The iron contained in the native globin solution (0·50 μg. per ml.) has been subtracted from the ESI values found.

	Total iron	ESI_{O_2}	ESI_{CO}	ESI_{N_2}
μg. per ml.	7·7	0·71; 0·56	0·74	0·50

Finally we have investigated the claims of Barkan & Berger [1930] that the CO-inhibition of the detachment of iron in mixtures of CO-haemoglobin and oxyhaemoglobin is much larger than it ought to be, if it were proportional to the saturation of haemoglobin with CO, and that the CO inhibition persists even after the reconversion of CO-haemoglobin into oxyhaemoglobin. This, indeed, would prove that the CO causing the inhibition of the iron splitting is bound to a compound different from haemoglobin. The experiments of these authors are, however, open to criticism. They claim to get the same results whether they add a known amount of CO dissolved in water to a solution of oxyhaemoglobin, or mix solutions of oxyhaemoglobin and CO-haemoglobin (saturated with CO) of about 1·5% haemoglobin concentration (blood diluted ten times) in the required proportions. This cannot be correct, since 100 ml. of 1·5% CO-haemoglobin solution contain 2 ml. of CO bound to haemoglobin, but at 15° at least 2·5 ml. CO in physical solution, and probably more, since this figure is based on the solubility in water, and that in protein solution is likely to be higher. The CO-haemoglobin content in the mixtures was not determined, and it is obvious that the mixtures must have contained a much greater proportion of CO-haemoglobin than Barkan & Berger assumed from the proportion in which the solutions had been mixed. There is, therefore, no evidence that the presumptive disproportionality between the degree of CO-inhibition of the iron splitting and the CO-haemoglobin concentration really exists. We can also not confirm the second claim. While the authors still find a considerable CO-inhibition of the iron detachment after 48 hr. of vigorous aeration of a CO-haemoglobin solution, we found that after 24 hr. aeration the ESI had returned to its original value.

The increase of ESI during choleglobin formation

During the coupled oxidation of haemoglobin and ascorbic acid the ESI of the solution rises simultaneously with the increase in absorption at 630 mμ due to choleglobin. In a series of experiments we have measured the ESI of choleglobin solutions and have compared the increase in ESI with the choleglobin concentration estimated spectrophotometrically as described in Part 3.

The choleglobin solutions contained 50–100 mg. ascorbic acid per 100 ml. From the results reported above it can be deduced that haemoglobin solutions containing ascorbic acid in this concentration yield ESI of about 1·5 % of the total iron. This value was therefore subtracted from the ESI found in our choleglobin solutions. The values thus obtained are given as ΔESI in Table 4. The procedure of subtracting the initial ESI is justified, even if the ESI or part of it were attributable to choleglobin, since the average absorption of CO-protohaemochromogen at 630 mμ has also been subtracted from the absorption values at 630 mμ for the estimation of the choleglobin content.

The values of choleglobin (including cholehaemochromogen in the 80 min. experiments at 37° in Table 4 are in percentage of initial haemoglobin and those of ΔESI in percentage of total iron, while in the bottom row (green pigment) the first figure means cholehaemochromogen in percentage of total haemochromogen (proto + chole) and the second figure ESI in percentage of total iron.

Table 4. *ESI from choleglobin*

Conditions of experiment	Time of incubation min.	Choleglobin	ΔESI	Ratio ΔESI : choleglobin
25°, *p*H 8·5	90	28·3	23·9	0·84
	120	42·3	28·4	0·67
37°, *p*H 8·5	15	17·9	11·7	0·65
	30	33·3	19·5	0·59
	80	45·5	23·8	0·52
37°, *p*H 7·2	30	14·1	12·2	0·86
	80	34·6	21·5	0·62
Green pigment	—	77·3	51	0·66
				Average 0·66

The increase of ESI under various conditions is evidently proportional to the amount of choleglobin formed, but the yield of ESI is only 66 % of that expected theoretically from the amount of choleglobin. The reason for this discrepancy is that a part of the cholehaematin remains unsplit and is still contained as such in the precipitate of denatured protein. If this precipitate is carefully washed free from trichloroacetic acid and redissolved in *N*/10 NaOH, a spectrophotometric determination of the absorption at 630 mμ of the CO-haemochromogen shows that 31 % of the cholehaematin has remained unsplit in this precipitate. It is possible that this observation means that choleglobin is not homogeneous and consists of two compounds, one easily split by acids and one that is not easily split, but the incomplete splitting cannot be considered as definite evidence against uniformity. We have observed similar incomplete splittings with verdohaemochromogen [Lemberg & Wyndham, 1937].

CO does not inhibit the detachment of iron from choleglobin solutions. In a few experiments a slight inhibition by CO (10–20 %) was found, but this is within the experimental range of error and in other experiments ESI_{CO} was found higher than ESI_{O_2}; usually both were equal (e.g. ESI_{O_2} 14·8 μg. per ml.,

ESI_{CO} 15·8, ESI_{CO} in presence of $Na_2S_2O_4$ 15·6). Even after the ascorbic acid had been removed by prolonged dialysis CO had no effect (ESI_{O_2} 18·9% of the total iron, ESI_{CO} 19·4%, ESI_{CO} in presence of $Na_2S_2O_4$ 18·4%). A solution of cyanmetacholeglobin (cf. Part 2) yielded 8·9% ESI_{O_2}, 7·3% ESI_{CO} and in presence of $Na_2S_2O_4$ without CO 9·6%, with CO 10·0% ESI, all in percentage of total iron. All these differences lie within the range of experimental error.

Discussion

From their observation that CO decreases the yield of labile iron from laked erythrocytes or oxyhaemoglobin solutions to about one-third of its value, Barkan & Schales have concluded that the ESI arises from a bile pigment-haemoglobin (pseudohaemoglobin). They assume that the iron detached by acid splitting in air, but not in CO, i.e. $ESI_{O_2}-ESI_{CO}$ (about 65% of the ESI and 3·5% of the total iron), is derived from a bile pigment-oxyhaemoglobin (EO_2), EO_2 being easily split by acids in contradistinction to the CO compound (ECO). The iron that is detached even in the presence of CO (ESI_{CO}), about 35% of ESI and 1·5% of the total iron, they believe to be the ferric form of the same bile pigment haemoglobin (E′), since they observed that it can be partially converted into the CO-inhibitable form by reduction with $Na_2S_2O_4$.

In our experiments described above we have been able to confirm the greater part of Barkan's experiments with the exception of the presumptive conversion of E′ into ECO by reduction and CO, for which we do not find conclusive evidence, and their observation that the CO inhibition of the detachment of iron occurs under conditions in which haemoglobin is no longer combined with CO.

We have shown that a small yield of biliverdin can indeed be obtained from erythrocytes and haemoglobin solutions by treatment with acids, and this lends support to the view that at least part of the ESI is attributable to a bile pigment-haemoglobin. The quantitative experiments described in Part 5 indicate, however, that the concentration of this bile pigment-haemoglobin in blood or haemoglobin preparations is too small to account for more than a small part of the ESI.

There are in fact, considerable difficulties in accepting Barkan's deductions about the nature of the ESI. The effect of CO on ESI is not specific, and Barkan explains the decreasing effect of inert gases on the yield of ESI by the assumption that only EO_2 (oxypseudohaemoglobin) and E′ (pseudomethaemoglobin) undergo splitting, while the iron in E (reduced pseudohaemoglobin) as well as in ECO (carboxypseudohaemoglobin) is resistant to acid. For chemical reasons this does not appear likely. Experience with haemoglobin derivatives shows that CO-haemoglobin and reduced haemoglobin are much more readily split than oxyhaemoglobin or methaemoglobin. It is not impossible that the properties of pseudohaemoglobin derivatives are exactly opposite to those of haemoglobin derivatives. Experience with known bile pigment-haematin compounds, e.g. verdohaemochromogen, shows that the ferric and ferrous forms are both split by acid, and that CO, while combining, does not prevent the splitting (cf. Part 5). Choleglobin appears to behave in the same manner.

Barkan & Berger [1928] describe an experiment, in which they acidified the haemoglobin under hydrogen and then aerated the solution. The amount of ESI found under these conditions was not larger than without aeration (equal to ESI_{CO}). In order to explain this result the authors are forced to make the unlikely assumption that E does not unite with O_2 in acid solution. In the presence of acid, E must be split to pseudohaem and no acid haem has been observed that is not readily autoxidizable to acid haematin, whether in the class

of porphyrin-haematins or bile pigment-haematins. The final product obtained under the conditions of the experiment ought to be, therefore, the same as from EO_2 and E′, and the ESI found ought to be equal to ESI_{O_2}, not to ESI_{CO}.

Several authors [Taylor & Coryell, 1938; Ammundsen, 1939] have recently confirmed earlier observations of Klumpp [1935] that a difference of about 5% exists between the O_2 capacity of blood and its CO capacity in the presence of $Na_2S_2O_4$. This indicates the presence of ferric haemoglobin or ferric bile pigment-haemoglobin in normal blood. From magnetochemical observations Taylor & Coryell also conclude that the substance combining with CO in the presence of $Na_2S_2O_4$ but not combining with O_2 is a ferric compound. Barkan & Schales [1939] mention the results of Ammundsen as being explained by the presence of 'pseudohaemoglobin', but this is evidently only partly true. While E+E′ form 5% of the total blood iron, only E′ (1·5%) combines with CO in presence of $Na_2S_2O_4$ and not with O_2, E (3·5%) would combine with both and thus not contribute to the difference of the gas capacities. The existence of this difference, therefore, supports only the assumption that E′ is the ferric form of a bile pigment-haemoglobin, and again E can explain only a fraction of the difference found between the O_2 and CO capacities.

The whole problem is evidently far from being satisfactorily solved, and we offer, therefore, an alternative explanation which, while not fully satisfactory in all respects, appears to us more likely and in better agreement with the observed facts. We shall discuss separately the CO-inhibitable part of the ESI ($ESI_{O_2}-ESI_{CO}$, attributed by Barkan to EO_2) and the CO non-inhibitable part (ESI_{CO}, attributed by Barkan to E), since we do not believe that there is evidence for a chemical relationship of the two forms. There is no evidence that the effect of CO on the blood ESI is specific and that CO acts in a way different from any other inert gas. The experiments indicate solely that presence of O_2 is necessary for setting free of the iron from E. The simplest explanation appears to us to be that the CO-inhibitable iron fraction ($ESI_{O_2}-ESI_{CO}$) is formed from oxyhaemoglobin by an oxidation process during the action of acid on haemoglobin. Recently several authors have observed that the O_2 set free from oxyhaemoglobin by denaturation has strongly oxidative power. Holden [1936; 1937] and Rawlinson [1939] have shown that the globin part of oxyhaemoglobin undergoes oxidation during the denaturation. Lemberg & Legge [1938] found that the O_2 set free from oxyhaemoglobin by acids oxidizes ascorbic acid. In Part 5 it will be shown that under the same circumstances biliverdin is oxidized to biliviolin. Engel [1939] has recently observed that some plasma bilirubin is oxidized during the precipitation of oxyhaemoglobin by alcohol, and that it can be protected against this oxidation by ascorbic acid. In the same way ascorbic acid protects biliverdin from being oxidized during the acid denaturation of oxyhaemoglobin. It is only necessary to assume that the oxidative effect can be turned against the prosthetic group of the haemoglobin molecule as well as towards the globin part. It has already been suggested by Lintzel & Radeff [1928] that ESI is derived from haemoglobin and that the prosthetic group is partially destroyed by the action of acid; the authors' claim is based on their observation that the colour of the acid haematin derived from oxyhaemoglobin is several per cent less in intensity than from the same amount of carboxyhaemoglobin. Barkan & Berger [1930] have accumulated material against this hypothesis, but their arguments are not fully convincing, although Lintzel & Radeff's observation may be explained by a different colloidal state of the 'acid haematins' derived from oxyhaemoglobin and CO-haemoglobin. Some of Barkan's arguments, e.g. the fact that the detachment of ESI is irreversible though incomplete, and the lack of formation

or porphyrin, do not speak against our hypothesis. His experiments with mixtures of CO-haemoglobin and oxyhaemoglobin are open to the criticism levelled above against his method of determining the ratio of the two compounds. It is doubtful, whether the slight increase of the ESI percentage in haemoglobin after partial adsorption on alumina, observed by Barkan [1936], is actually due to a preferential adsorption of haemoglobin on alumina; if one remembers the ease with which small amounts of choleglobin are obtained from haemoglobin, it is quite likely that a slight oxidation of haemoglobin to choleglobin occurs during the adsorption experiments; Barkan has omitted to show that the haemoglobin recovered from the alumina contained less ESI.

The majority of Barkan's and our observations can be explained satisfactorily on the assumption that ESI is derived from haemoglobin by oxidation during the acidification. Inert gases or CO would prevent this reaction by transforming oxyhaemoglobin into reduced haemoglobin or CO-haemoglobin, while ascorbic acid would prevent the oxidative removal of iron from the haemoglobin molecule by being oxidized instead, in the same way as it protects added bile pigment. Once the acid denaturation has taken place under exclusion of O_2, aeration would no longer cause oxidative removal of iron, in agreement with the experiment of Barkan & Berger mentioned above. The transformation of oxyhaemoglobin into methaemoglobin does not decrease the yield of ESI [Barkan & Berger, 1928], since the oxidative reaction setting free the iron would occur during the methaemoglobin formation.

One observation in our experiments would still remain unexplained, namely the presence of the usual percentage of ESI in solutions of synthetic methaemoglobin, but this observation too speaks against the theory of Barkan and favours the assumption that some ESI is derived from haemoglobin itself, since any bile pigment-iron compounds have been excluded by the method of preparation.

While the greater part of ESI, about two-thirds, would thus have to be considered as an artefact, there would still remain the one-third (ESI_{CO}) which can be split off even in inert gases. Here our experiments support Barkan's view of the bile pigment-haemoglobin nature of at least a part of this fraction. In Part 5 we shall describe the isolation of small amounts of biliverdin and bilivіolin from red cells. We have not yet been able to decide whether or not the detachment of iron during the acid denaturation of haemoglobin, leads to formation of bile pigment. If the porphyrin part of the haemoglobin molecule is oxidized to substances other than bile pigment, the yield of bile pigment indicates that almost the total ESI_{CO} fraction is bile pigment-haemoglobin. Otherwise a part of the bile pigment found would also be an artefact, certainly, however, not all, since in the presence of ascorbic acid biliverdin was obtained from red cells.

A certain part of E′ must also be the blood catalase. We have recently proved [Lemberg, Norrie & Legge, 1939] that a bile pigment-haematin group occurs in catalase. A simple calculation shows, however, that the blood catalase constitutes only a small fraction of the total E′. The monomolecular reaction constant k of rabbit's blood catalase multiplied by the dilution is 1500 (own unpublished experiments). If the catalase factor of pure catalase ($=k/g.$ catalase in 50 ml.) is 35,000, this corresponds to a catalase concentration of 86 mg. catalase in 100 ml. of blood. Catalase contains one equivalent of bile pigment-haematin iron in a molecule of mol. wt. 248,000. The ESI derived from blood catalase is, therefore, about 20 μg. per 100 ml. of blood, while the total ESI_{CO} (E′-iron) is about $\frac{15{,}000{,}000 \times 55{\cdot}8 \times 1{\cdot}5}{16{,}700 \times 100} = 750$ μg. per 100 ml. Catalase forms,

therefore, less than 3% of the E. Barkan & Schales [1936] have, indeed, separated catalase from E′ by adsorption on alumina, and with the slight qualification that their separation means separation of catalase from the main bulk of E′, their experiments are in agreement with the above calculation.

Summary

The findings of Barkan & Schales are confirmed that blood and haemoglobin solutions contain 5–6% of the total iron in the form of 'labile iron' (ESI_{O_2}) and that about two-thirds of this ($ESI_{O_2}-ESI_{CO}$) are split off only in the presence of O_2 and in the absence of reducing substances while the remaining third is split off also in inert gases and CO (ESI_{CO}).

In contradistinction to Barkan & Schales we explain the fraction $ESI_{O_2}-ESI_{CO}$ as an artefact, arising by oxidation of the prosthetic group of the haemoglobin molecule by the O_2 evolved from oxyhaemoglobin by acids.

The fraction ESI_{CO} is at least partially attributable to a bile pigment-haemoglobin which gives rise to bile pigments when treated with acids. Only a small part of ESI_{CO} is due to the blood catalase.

The increase of labile iron occurring during the coupled oxidation of haemoglobin and ascorbic acid is proportional to the concentration of choleglobin formed, the latter being estimated spectrophotometrically. About 66% of the choleglobin iron is split off by 16 hr. incubation with $N/10$ hydrochloric acid, while the remainder is found as unsplit cholehaematin in the precipitate of denatured protein. Neither CO nor reducing substances appear to inhibit the acid splitting of choleglobin.

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36. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

5. STUDIES ON THE FORMATION OF BILE PIGMENTS FROM CHOLEGLOBIN AND VERDOHAEMOCHROMOGEN AND ON THEIR ISOLATION FROM ERYTHROCYTES[1]

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In a preliminary paper [Lemberg, Legge & Lockwood, 1938] we have already reported the isolation of biliverdin (dehydrobilirubin) and of biliviolin from choleglobin solutions. Under remarkably mild conditions haemoglobin is thus transformed into bile pigments; these conditions are such as are found in the cells of organs forming bile pigment, except that acid was used for the removal of the iron. It will be shown in the present paper that the breakdown of choleglobin to bile pigments with the removal of iron probably occurs in the later stages of the reaction with ascorbic acid at a physiological *p*H.

We describe here in greater detail the bile pigments obtained by acid splitting from choleglobin and verdohaemochromogen. The hope of gaining information on the constitutional differences between cholehaem and verdohaem by the study of the splitting products was not fulfilled owing to secondary reactions by which the primary splitting products are altered and owing to the complicated nature of the mixtures of bile pigments thus formed.

Great difficulties had, therefore, to be overcome in working out quantitative methods enabling us to estimate the yield of bile pigment from the bile pigment-haematin compounds. This was, however, achieved and we can now correlate the yield of bile pigments obtained from choleglobin solutions by splitting with acids with the choleglobin concentration of these solutions.

In the above-mentioned preliminary publication we had also reported the isolation of bile pigments from erythrocytes. In the present paper we describe these experiments in greater detail.

Experimental

Preparation of biliverdin and biliviolins[2] from haemoglobin by coupled oxidation with ascorbic acid

In each of 12 flat-bottomed culture flasks 50 ml. of a 1·5% solution of crystalline horse oxyhaemoglobin, buffered to *p*H 8·5 with hydrogen phosphate,

[1] This work was carried out under a grant from the National Health and Medical Research Council of Australia.

[2] According to Siedel (*Angew. Chemie*, **52**, 38 [1939]) our compounds would be 'bilipurpurins', not 'biliviolins'. Siedel restricts the name 'biliviolins' to a very similar class of substances, probably isomeric with bilirubins, one of which e.g. is formed by the oxidation of mesobilinogen by ferric chloride; for the pigments of an oxidation stage higher than the biliverdins he uses the term 'bilipurpurins'. The extraordinary similarity of the properties of 'biliviolins' and 'bilipurpurins', and the fact that the latter comprise again several pigment classes, makes it appear more practical to maintain the generic name biliviolins, for all these pigments, and to distinguish them by figures and letters, until more is known about their chemical constitution. The term also tallies better with the actual colour of the pigments.

were incubated for 2 hr. with 50 mg. of ascorbic acid at 37°. The supernatant solution was poured off from the green pigment and poured into 2 vol. of glacial acetic acid. After 15 min. at room temperature this solution was poured into 2 vol. of ether (freed from peroxide over sodium) and the protein removed together with the greater part of the acetic acid by repeated washing with dilute sodium acetate solution and finally with water, avoiding shaking in the initial stages.

Biliverdin was extracted from the ethereal solution by repeated extractions with a few ml. of 5% HCl. After a few hours green flocks of biliverdin hydrochloride settled down; they were collected by filtration and washed on the filter with water. The mother liquor contained a biliviolin. The biliverdin hydrochloride was dried on the filter in a vacuum desiccator and transformed into the dimethyl ester of biliverdin in the usual way. The ester was brought once more into ether and finally recrystallized from methyl alcohol. 20 mg. of blue-green needles. M.P. (on the copper block) 216°, mixed M.P. with an ester prepared from bilirubin (M.P. 212°) was 214°.

After the extraction with 5% HCl the ether still contained a weakly basic biliviolin and protohaemin. The former was extracted with 20% HCl. Haemin was then extracted with dilute ammonia and transformed into the pyridine-haemochromogen by pyridine and $Na_2S_2O_4$. Only the absorption bands of proto-haemochromogen could be observed. Cholehaematin must, therefore, have been transformed into the bile pigments, except that part of it which remains unsplit in the protein precipitate. The biliviolin present in the 20% HCl extract was taken up by chloroform from the diluted solutions. The chloroform solution was washed to neutrality with water. It was then red-violet and showed a rather diffuse absorption band at 562 mμ and a second weaker band at 520 mμ.

A phenomenon characteristic of the different biliviolins is their hydrochloride formation, when a neutral chloroform phase containing the pigment is shaken with an aqueous phase containing dilute HCl. The absorption spectrum changes from that of the neutral compound to that of the hydrochloride with an absorption band in the orange part of the spectrum, while the colour changes from pink or red-violet to blue-violet or blue, the colour change being more marked in daylight than in artificial light. This change depends on the concentration of the acid in the aqueous phase and also to a remarkable degree on the temperature, a higher temperature favouring salt formation. This can only be explained by the assumption that biliviolins occur in two tautomeric forms, the more strongly basic form being unstable and favoured by higher temperature.

The biliviolin obtained from the 20% HCl extract behaved in this chloroform-HCl test as indicated by Table 1. We shall call it biliviolin III*a*. Its hydrochloride in aqueous solution was violet, in chloroform or in methyl alcohol in daylight almost blue, in artificial light violet. Its aqueous solution showed a broad absorption band with its centre at 596 mμ, with its maximum, however, somewhat more towards the red at about 604 mμ.

Table 1. *Chloroform-HCl test of biliviolin III a*

Temperature	1%	5%	10% HCl
0°	Neutral	Neutral	Partly acid
20°	Neutral	Neutral	Acid
60°	Neutral	Partly acid	Acid

In chloroform the band was at 622 mμ. The zinc salt in methyl alcohol was blue-green with red fluorescence and a two-banded absorption spectrum (I 646, II 581 mμ; intensities: I, II).

Table 2. *Chloroform-HCl test of biliviolin II b*

Temperature	1%	5%	10% HCl
0°	Neutral	Partly acid	Acid
20°	Neutral	Acid	Acid
60°	Acid	Acid	Acid

A second biliviolin was isolated in smaller yield from the mother liquor of biliverdin hydrochloride. It showed a different behaviour in the chloroform-HCl test (Table 2). We shall call it biliviolin II*b*. The biliviolins can either be derived directly from choleglobin, or they may be products of a secondary oxidation of biliverdin. If about 200 μg. biliverdin are dissolved in dilute ammonia and added to a 0·7% oxyhaemoglobin solution and this mixture is treated with glacial acetic acid, the biliverdin is transformed into biliviolin, which according to its properties is biliviolin II*b*. The same biliviolin had been previously obtained by oxidizing the complex zinc salt of biliverdin with two atoms of iodine per mol. and by exposing the unstable biliviolin II*a*, set free from its zinc salt, to atmospheric O_2 [Lemberg & Lockwood, 1938, and unpublished experiments]. It is therefore likely that this biliviolin arises by secondary oxidation of biliverdin during the choleglobin splitting with acids.

The choleglobin solutions contain ascorbic acid, and if ascorbic acid in the same concentration (50 mg. per 100 ml.) is added to the mixture of biliverdin and oxyhaemoglobin, there is practically no oxidation of biliverdin to biliviolin. Even in the presence of excess of ascorbic acid or of CO, biliviolin III*a* was always found to accompany biliverdin in the splitting products of choleglobin. Ascorbic acid protects biliverdin from oxidation during the action of acid on oxyhaemoglobin (cf. Table 6), but does not reduce biliviolins to biliverdin. These experiments make it likely that the biliviolin III*a* is a genuine splitting product of choleglobin. It will be shown below that verdohaemochromogen yields also biliviolin besides biliverdin in the presence of ascorbic acid, and that the primary split-product of verdohaemochromogen is apparently oxidized by ferric iron to biliviolin. This biliviolin, however, is biliviolin II*b*, the same that arises by oxidation of biliverdin, while the biliviolin III*a* from choleglobin is different.

The same split products were obtained from choleglobin if the method of splitting was varied, e.g. if an acetic acid-ethyl acetate mixture (1:2) was used, or acetic acid containing HCl or if the splitting was carried out at 100°. With acetic acid and hydrazine hydrate or with ferrous acetate in acetic acid-HCl, however, only biliverdin was isolated. In these reactions no trace of the hydroxyporphyrin described below was observed.

Split products of 'green pigment'

Cholehaemochromogen obtained from choleglobin by alkali denaturation gave the same split products as choleglobin. The 'green pigment', however, which precipitated in the further course of the coupled oxidation of haemoglobin and ascorbic acid behaved somewhat differently in spite of the identity of its absorption properties with those of the first-mentioned cholehaemochromogen. The yield of ether-soluble split products was very small, the major part of the pigment remaining in the denatured protein which precipitated in the aqueous phase on addition of sodium acetate solution. Not only the bile pigments, but also the haemin from the admixed protohaemochromogen were thus largely lost. The kind of linkage of the prosthetic groups with the protein in the 'green pigment' is evidently quite different from that in the native and in the alkali-denatured chromoproteins. This is the reason why earlier observers failed to

isolate bile pigments. The green, amyl alcohol-soluble pigments, obtained by treating 'green pigment' with hot mineral acids [Edlbacher & v. Segesser, 1937] are still compounds of peptide nature, resulting from partial decomposition of the firmly attached protein groups. While their positive zinc acetate-iodine reaction (red fluorescence and absorption band at 636 mμ) proves their close relationship to biliverdin, their solubility properties reveal the presence of large hydrophilic groups. They are insoluble in ether and absolute alcohol, but easily soluble in water.

Nevertheless small amounts of ether-soluble bile pigments were obtained by splitting green pigment with 66 % acetic acid—and these were the same as those isolated from choleglobin. A slightly larger yield of biliverdin was obtained, when 'green pigment' was dissolved in 1 % ascorbic acid (no reduction occurs, the absorption band being that of acid ferric cholehaemochromogen at 610 mμ) and reduction to the ferrous haemochromogen caused by the addition of a minimum amount of $Na_2S_2O_4$ before the addition of acetic acid.

While acetic acid splitting thus yielded the same products as from choleglobin, though in much smaller yield, another split product of different nature was found, when the splitting was carried out with a ferrous acetate-acetic acid-HCl mixture or with glacial acetic acid in the presence of hydrazine hydrate. From the ether solution 0·5 % HCl extracted biliverdin, and 5 % HCl protoporphyrin and small amounts of a compound with a sharp absorption band at 627 mμ. Somewhat larger amounts of this substance were contained in the 20 % HCl extract. The yield of all split products was again small. The new compound was brought back into ether by partial neutralization of the 20 % HCl extract and separated from the last traces of protoporphyrin by repetition of the HCl fractionation. The acid solution was green without fluorescence, while the dirty yellow neutral solution in ether-acetic acid showed red fluorescence. This solution showed four absorption bands: I 684, II 592, III 544, IV 504 mμ (intensities: I, III, IV, II). The Willstätter number was about 14. The copper complex salt showed the following spectrum: I 628, II 532 mμ (intensities I, II).

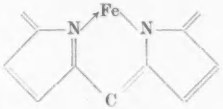
The properties of the substance show that it is different from the hydroxyporphyrin isolated by Lemberg, Cortis-Jones & Norrie [1938] from its haematin which was observed as an intermediate product in the formation of verdohaemochromogen from pyridine haemochromogen with ascorbic acid. It appears, however, to bear a close resemblance to the oxymesoporphyrins of Fischer *et al.* [1927; 1930], substances of uncertain constitution arising by the action of H_2O_2 on mesoporphyrin. Probably it is the corresponding proto compound, the absorption bands lying more towards the infra-red.

The fact that this hydroxyporphyrin cannot be obtained in the same way from choleglobin solutions, makes it unlikely that its haematin compound should be formed as intermediate product in the formation of choleglobin, or that it should be derived from cholehaematin itself. Some, though not all of Fischer's analyses indicate the presence of two oxygen atoms in addition to the four present in the two carboxylic groups of mesoporphyrin. Probably our new compound is a dihydroxyprotoporphyrin, the haematin of which is produced by a side reaction involving the oxidation of a second methene group before the ring is opened at the methene group first oxidized.

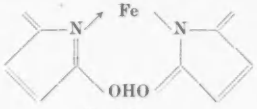
Bile pigments from verdohaemochromogen and verdohaematin

Verdohaemochromogen was shown by Lemberg [1935] to be transformed into biliverdin by hot methyl alcoholic HCl. Libowitzky & Fischer [1938] have recently claimed that the verdohaemochromogen of the copro series is not split

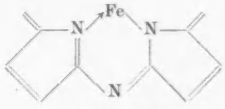
by a mixture of equal parts of glacial acetic acid and conc. HCl. From this observation they conclude that the 'green haemin' is not a haemochromogen, but a free ferric haematin, probably not of bile pigment-haematin nature. Warburg & Negelein have, however, already shown that the typical three-banded absorption spectrum disappears when the pyridine is washed away from the chloroform solution; it also disappears when the haemochromogen is split by acetic acid or by alkali. We can show also (cf. Table 7 below) that verdohaemochromogen can be split to bile pigments with good yield under much milder conditions than those applied by Fischer. The data collected in Table 7 show that if there is any difference in the reactions of ferric verdohaematin and verdohaemochromogen with acids, the former is split more easily. Libowitzky & Fischer base their assumption also on the observation that hydrazine hydrate destroys the typical absorption spectrum. That hydrazine does not destroy 'green haemins' by reduction, is, however, evident from the fact that it is used in their preparation from haemins. Verdohaemochromogens are unstable in all strongly alkaline solutions which transform them into yellow compounds. We have recently obtained further conclusive evidence [cf. also Holden & Lemberg, 1939] for the formula of verdohaemochromogen as an *iso*biliverdin-haemochromogen with opened ring by condensing it with ammonia and transforming it thus into the haemochromogen of an azaporphyrin with nitrogen-closed ring (unpublished experiments).



Protohaem



Verdohaem



Aza-haem

Verdohaemochromogen appears to us best formulated as a resonance structure with a hydrogen linkage between oxygen atoms closing the ring.

We have now subjected verdohaematin, verdohaemochromogen and verdoglobin to the same mild splitting conditions as were used for choleglobin above. Quantitative data will be given later; here we describe the splitting products. Again under all conditions mixtures of biliverdin and biliviolin were obtained (cf. Table 6), whether an alkaline solution of ferric verdohaematin or a solution of the haemochromogen in pyridine was treated with 66% acetic acids. On keeping at room temperature biliviolins were obtained predominantly, while on short boiling biliverdin became the main product. Mainly biliverdin was formed when the splitting was carried out in the presence of ascorbic acid. Glacial acetic acid plus hydrazine hydrate and ferrous acetate in acetic acid-HCl in the heat produced biliverdin only.

For the isolation of the biliviolins 2 vol. of glacial acetic acid were added to the freshly prepared solution of verdohaemochromogen in 2% NaOH. After 15 min. at room temperature the solution was diluted with an equal volume of ether. Washing with water removed some unsplit verdohaematin and a little biliverdin. From the red-violet ether solution 2% HCl extracted two biliviolins, one of which (the main product) passed from 2% HCl into chloroform, while the second was not extracted by chloroform. In the chloroform-HCl test the first substance behaved as indicated in Table 3.

The acid aqueous solution showed an absorption band at 595 mμ, the acid chloroform solution one at 614 mμ. The neutral chloroform solution had an absorption spectrum with two bands of about equal intensities (I 552, II 512 mμ).

Table 3. *Chloroform-HCl test of biliviolin II b from verdohaemochromogen*

Temperature	1%	5%	10% HCl
0°	Neutral	Neutral	Acid
20°	Neutral	Partly acid	Acid
60°	Partly acid	Acid	Acid

The zinc salt was blue-green with strong red fluorescence and absorption bands at 640 and 585 mμ, the former being far stronger. In spite of slight differences in the chloroform-HCl tests (cf. Tables 2 and 3) this violin is certainly identical with biliviolin II*b*.

The second biliviolin was brought into chloroform by neutralization with sodium acetate. The neutral solution had a somewhat weaker second band than that of biliviolin II*b*. 1% HCl extracted it completely from chloroform. The centre of the absorption band of its hydrochloride was at 592 mμ. This biliviolin, of which only traces are obtained, appears to be identical with that formed as a primary product by dehydrogenation of the biliverdin zinc salt with iodine (biliviolin II*a*; unpublished experiments, and Lemberg & Lockwood [1938]).

The original ether solution was further successively extracted with 5, 10 and 20% HCl. All these extracts contained violin. While the violin in the 5% HCl extract was also biliviolin II*b*, the other fractions contained a third biliviolin of weaker basicity. The absorption bands of the neutral solution in chloroform were at 554 and 514 mμ; those of the acid chloroform solution at 617 mμ. It differs from the biliviolin III*a* isolated from choleglobin by having absorption bands more towards the blue. We shall call it, therefore, biliviolin III*b*. Its behaviour in the chloroform-HCl test (Table 4) does not differ significantly from that of biliviolin III*a*.

Table 4. *Chloroform-HCl test of biliviolin III b from verdohaemochromogen*

Temperature	1%	5%	10% HCl
0°	Neutral	Neutral	Largely acid
20°	Neutral	Neutral	Acid
60°	Neutral	Partly acid	Acid

The comparatively strongly basic biliviolin II*b* forms the main bulk, while from choleglobin the weakly basic biliviolin III*a* was formed predominantly.

Methods for the quantitative estimation of biliverdin and biliviolin

The methods for quantitative determination of bile pigments detached from choleglobin or verdohaemochromogen solutions were based in principle on the methods of isolation described above. For an exact determination of the bile pigment yield from these substances the biliviolin cannot be neglected. In the later phases of the coupled oxidation, however, biliverdin prevails. Biliverdin was also obtained as main product from catalase [Lemberg, Norrie & Legge, 1939]. We have therefore worked out a microcolorimetric method for the determination of biliverdin alone, which is simpler and requires less material (method I). For the determination of biliviolin as well as biliverdin spectrophotometric methods were developed. In the first of these (method II) biliverdin and biliviolin are separated by the Willstätter method of fractional HCl extraction from ether and determined separately, while in the second (method III) both are extracted together and their concentrations are calculated from the extinction coefficients at the wave-lengths of the maximal extinctions of biliverdin and biliviolin at 675 and 595 mμ respectively. Method II is suitable for deter-

mination of the bile pigments from choleglobin solution, since the weakly basic biliviolin III*a* is easily separated from biliverdin, and unsuitable for the determination of the bile pigments from verdohaemochromogen, with which such a separation is difficult owing to the relatively strong basicity of biliviolin II*b*. Here and for the study of the recovery of biliverdin added to oxyhaemoglobin solutions, in which also biliverdin II*b* is formed, method III was applied.

Method I. 5 ml. of the choleglobin solution are pipetted into 10 ml. of glacial acetic acid and after 15 min. at room temperature the mixture is poured into 15 ml. of peroxide-free ether in a narrow 50 ml. separating funnel. 5 ml. of saturated sodium acetate solution are added, the tube once inverted and 10 ml. of water added. After removal of the aqueous phase the protein precipitate suspended in the ether is removed together with the main bulk of acetic acid by washing with 10 ml. of dilute sodium acetate solution. Vigorous shaking must be avoided during the initial stages. The ether solution is then washed 2–3 times with water, the last time with shaking. It is essential to have the last trace of protein removed, but on the other hand to leave enough acetic acid in the ether to prevent precipitation of haematin. The first two sodium acetate extracts are overlayered once more with a few ml. of ether and saturated ammonium sulphate solution is added, until the protein comes out in green flocks at the interface and the aqueous layer becomes colourless. The latter is removed and the ether solution is poured off from the protein, washed with dilute sodium acetate solution and added to the main ether extract.

Biliverdin is then extracted by 2 ml. of 2% HCl, followed by two further extractions with 2 ml. of 5% HCl each. The united extracts are made up to 10 ml. with alcohol and matched colorimetrically with a biliverdin standard containing 100 μg. biliverdin in 10 ml. 40% alcohol with an HCl content of 2·5%. The standard is prepared freshly each day from a stock solution of 10 mg. pure crystalline biliverdin in 100 ml. alcohol, which is kept in the dark. During 6 weeks the specific extinction coefficient (ϵ of an 0·1% solution) of the acidified solution at the maximum of absorption ($\lambda = 675$ mμ) diminished very little (from 47·1 to 46·5).

Method II. Biliviolins cannot be estimated colorimetrically, since their solutions are not stable enough to be used as standards, and other pigments which match the colour exactly are not available. A spectrophotometric method is therefore used. Biliviolins have not yet been obtained crystalline and their specific extinction coefficients are at present unknown. The purification of the biliviolin III*a* from choleglobin in sufficient quantities promised little hope of success. We have therefore used a fresh sample of biliviolin II*b*. This was isolated from the 5% HCl fraction obtained after splitting of verdohaemochromogen with acetic acid and extraction with ether. It was brought back into ether with sodium acetate and the acetic acid was removed by extraction with water. The evaporation residue of the ether solution was dried *in vacuo* and weighed. It was then dissolved in 2 ml. of alcohol and diluted to 10 ml. with 20% HCl. The specific extinction coefficient at the maximum of absorption (at 595 mμ) was found to be 46·0. This value is so close to that of the maximal specific extinction coefficient of biliverdin, that it can be reasonably assumed that the sample of biliviolin was pure, and also that the maximal extinction coefficient of biliviolin III*a* from choleglobin will not be very different from that of biliviolin II*b* and biliverdin. The value 46·0 for the specific extinction coefficient of biliviolin as hydrochloride at 595 mμ and the value 46·5 for that of biliverdin as hydrochloride at 675 mμ were used to convert spectrophotometric readings into terms of concentration.

For method II 50 ml. of a choleglobin solution obtained from 0·7 % haemoglobin are required. They are worked up as described for method I, the extraction of the bile pigments being carried out, however, in a different manner and with relatively smaller volumes of HCl in order to get sufficiently strong solutions. 10–15 ml. of 5 % HCl are used for the extraction of biliverdin and the same volume of 20 % HCl for the extraction of biliviolin for samples of choleglobin solutions obtained by a short incubation of haemoglobin with ascorbic acid, while for samples of longer incubation time 25–30 ml. of the acids can be used. 5 ml. of alcohol are added and the final volume is measured. In a layer of 4–5 cm. length these bile pigment solutions give extinctions in the optimal range of the spectrophotometer. The instrument was the Hilger Visible Spectrophotometer described in Part 2.

Method III. If the extinction coefficients of both biliverdin and biliviolin are known at two wave-lengths at which they differ considerably, the content of a mixture of both substances can be established by measuring the extinctions at these wave-lengths. The wave-lengths of the absorption maxima ($\lambda = 675$ and 595 mμ) are suitable. The specific extinction coefficients of biliverdin hydrochloride were found to be 46·5 at 675 mμ and 21·4 at 595 mμ, while those of biliviolin hydrochloride were 46·0 at 595 mμ and 6·0 at 675 mμ. If ϵ_{675} and ϵ_{595} are the extinction coefficients of the solution in 1 cm. depth at the wave-lengths 675 and 595 mμ the following two equations hold:

$$\mu\text{g. biliverdin per ml.} = 2{\cdot}98\ (7{\cdot}67\ \epsilon_{675} - \epsilon_{595})$$
$$\mu\text{g. biliviolin per ml.} = 10{\cdot}64\ (2{\cdot}17\ \epsilon_{595} - \epsilon_{675}).$$

For the determination with this method the ethereal solution is extracted twice with 5 ml. each of 5 % HCl and three times with 5 ml. each of 20 % HCl. After the addition of 5 ml. of alcohol to the united extracts the volume is measured and the extinction coefficients at 675 and 595 mμ are measured.

Table 5 shows that with choleglobin methods II and III give essentially the same results, while the differences are larger when the bile pigments from verdohaematin or verdohaemochromogen are determined; here method III is preferable.

Table 5. *Determination of bile pigments from verdohaemochromogen and choleglobin*

	Method II			Method III		
	μg. verdin	μg. violin	μg. bile pigment	μg. verdin	μg. violin	μg. bile pigment
Verdohaematin	52	104	156	33	134	166
Verdohaemochromogen + ascorbic acid	167	42	209	165	106	271
Choleglobin	950	251	1201	978	203	1191

Bile pigment yield from verdohaemochromogen

Experiments on the yield of bile pigments from verdohaematin compounds were carried out in order to examine in what yield bile pigment is isolated from these bile pigment-haematin compounds of established constitution under the conditions used for the splitting of choleglobin.

Verdohaemochromogen was either dissolved directly in 66 % acetic acid (*a*) or it was first dissociated to verdohaematin by dissolving it in 1 ml. of 2 % NaOH (*b*) or dissolved in 1 ml. of 20 % pyridine (*c*) and these solutions added to 2 ml. of glacial acetic acid. Finally a solution of verdohaematin in 1 % Na_2CO_3

was coupled with native horse globin and 6 ml. of this solution of ferric verdoglobin (*d*) were added to 12 ml. of glacial acetic acid. The bile pigments were isolated as from choleglobin, the unsplit verdohaematin being removed from the ether by repeated washing with water before the extraction of the bile pigments with HCl. Method III was applied to the determination and the results are given in Table 6.

Table 6. *Bile pigment yields after acid splitting of verdohaematin compounds under various conditions*

Additions to the solution before splitting	Split by	Time min.	Total bile pigment in % of theory: *a* Solid verdo-haemo-chromogen	*b* Verdo-haematin in alkali	*c* Verdo-haemo-chromogen in 20% pyridine	*d* Ferric verdo-globin	Biliverdin in % of total bile pigment: *a*	*b*	*c*	*d*
—	66% acetic acid	15	13·8	25·8	6·1	14·7	16	38	35	19
		60	18·5	32·5	13·7	—	25	44	18	—
5 mg. ascorbic acid	„	15	—	25·6	6·8	10·7	—	74	62	49
		60	25·3	34·3	12·2	—	58	89	55	—
5 mg. ascorbic acid, +CO	66% acetic acid under CO	15	—	17·0	—	12·1	—	63	—	46
		60	21·5	31·4	—	—	54	71	—	—
—	66% acetic acid + 1 ml. conc. HCl	60	19·3	—	—	—	28	—	—	—
—	66% acetic acid, boiling	2	39·5	22·6	23·7	10·4	70	55	87	58
—	5% HCl	60	—	33·0	—	—	—	65	—	—
5 mg. ascorbic acid	„	60	—	32·6	—	—	—	73	—	—

Under the mild conditions used for the splitting of choleglobin the iron from verdohaematin compounds is not detached completely, but already to the extent of 15–35% at room temperature and up to 40% by boiling acetic acid. Neither HCl, nor a mixture of acetic and hydrochloric acids increases the yield of bile pigments substantially. The degree of splitting varies a great deal with the conditions. If there is any difference between ferric verdohaematin and ferrous verdohaemochromogen in this regard, ferric verdohaematin is split more readily. The difference is, however, not large. Thus ferrous verdohaemochromogen was observed spectroscopically to persist in the 66% acetic acid in the presence of pyridine and ascorbic acid or of pyridine, ascorbic acid and CO, while its absorption spectrum rapidly disappeared in the acetic acid in the absence of ascorbic acid; the bile pigment yield was slightly higher in the absence of ascorbic acid. In the absence of excess pyridine CO reacts in the acetic acid with verdohaem when ascorbic acid is present, yielding a characteristic CO-compound with absorption bands at 611, 541 and 502 mμ (intensities: I, II, III); when ascorbic acid is absent, CO fails to form this compound. It is, therefore, CO-verdohaem with ferrous iron; pyridine converts it into verdohaemochromogen. Again the yield of bile pigment isolated from the ferrous CO-verdohaem is either the same or slightly less than from the brown ferric verdohaematin solutions obtained by the action of acetic acid on verdohaemochromogen. Verdoglobin is split by 66% acetic acid with a yield of 10–15% and neither ascorbic acid nor CO influences the yield significantly. Under all these conditions both biliverdin and biliviolin are obtained, predominantly verdin from ferrous and violin from ferric compounds. Boiling acetic and hydrochloric acids produce less violin from ferric

verdohaematin than acetic acid at room temperature. Biliviolin II*b* contains four hydrogen equivalents less than biliverdin and one would expect, therefore, a yield of 25 % of violin, if oxidation of biliverdin by the ferric haematin iron causes the formation of the biliviolin. Actually much more biliviolin is always found, although the difference in the percentages of biliverdin in total bile pigment from a ferrous and a ferric compound is roughly 25 %. Since biliviolin is not reduced by ascorbic acid, and biliverdin is not oxidized by the small concentrations of ferric acetate set free in the solutions, it is somewhat difficult to explain these results, unless it is assumed that the unstable *iso*biliverdin set free from verdohaematin compounds is more readily oxidized than biliverdin.

Recovery of biliverdin from mixtures with oxyhaemoglobin

For the determination of the bile pigment yields from choleglobin solutions, which always contain oxyhaemoglobin, it was essential to know to what extent biliverdin can be recovered by our methods from a solution of oxyhaemoglobin. A known amount of biliverdin dissolved in dilute ammonia was added to 50 ml. of an 0·7 % solution of horse oxyhaemoglobin. This amount of oxyhaemoglobin alone gave no detectable amounts of bile pigments. In the majority of the recovery experiments 50 mg. per 100 ml. of ascorbic acid were added, this being about the ascorbic acid concentration of the majority of choleglobin solutions. It has already been mentioned above that ascorbic acid prevents the oxidation of biliverdin to biliviolin during the treatment with acid. The mixture of oxyhaemoglobin, biliverdin and usually ascorbic acid was subjected to the treatment with 66 % acetic acid as described above, and the recovered bile pigment determined by method III.

Table 7 shows that, under the conditions of the determination of the bile pigment yield from choleglobin, between 80 and 90 % (average 84 %) of the added biliverdin can be recovered and that the percentage of recovery does not

Table 7. *Recovery of biliverdin from dilute oxyhaemoglobin solutions*

		In % of added biliverdin		
μg. biliverdin added	Ascorbic acid mg. per 100 ml.	Biliverdin	Biliviolin	Total bile pigment
62·9	50	79·0	2·2	81·2
63·5	50	79·0	0	79·0
95	50	90·7	0	90·7
189	50	95·9	0	95·9
222	50	83·0	0	83·0
222	50	68·5	0·5	69·0
262	50	90·8	0	90·8
408	50	83·0	1·9	84·9
952	50	75·0	0	75·0
62·9	—	0	85·8	85·8
95	—	2·9	86·4	89·3
				Average 84 %

depend on the amount of biliverdin in a range between 60 and 400 μg. per 50 ml., i.e. in the range of bile pigment yields obtained from choleglobin solutions. In the absence of ascorbic acid biliverdin is oxidized to biliviolin II*b* by the action of acid on oxyhaemoglobin, but the yield of biliviolin is the same as that of biliverdin obtained in the presence of ascorbic acid.

Bile pigment yield from choleglobin

We found that splitting at room temperature for 15 min. in 66 % acetic acid gave optimal yields of bile pigment from solutions of choleglobin. Heating, or treatment with the acid for a longer period, or the application of stronger acids, e.g. HCl, decreased the yield. Probably more of the bile pigment-haemoglobin is split, but also more is denatured in a way which prevents the removal of the bile pigment from the protein. In Table 8 the yields of bile pigments obtained from choleglobin solutions after various times of incubation of haemoglobin with ascorbic acid are compared with the amount of choleglobin determined spectrophotometrically. A superficial examination may lead to the assumption that the bile pigment is not derived from choleglobin, since there is evidently no proportionality between the amount of choleglobin and the yield of bile pigment. While the maximum of choleglobin concentration in the solution is reached in about 30 min. at *p*H 8·5 and in about 80 min. at *p*H 7·2, the maximum of the bile pigment formation occurs only after about 7 hr. incubation; for these calculations we have left out of consideration the cholehaemochromogen of the green pigment precipitate, since little bile pigment can be isolated from green pigment, at least in the first stages of its formation. It is assumed that one mole of choleglobin of mol. wt. 16,700 yields 584 g. bile pigment, 584 being the mol. wt. of biliverdin. The mol. wt. of biliviolin is somewhat higher, but the error introduced by this in the calculations, is insignificant. The data in Table 8 show that in the first stages of the reaction only 10–15 % of the bile pigment theoretically expected from the choleglobin content of the solutions is isolated, while later up to 70 % can be recovered. Choleglobin would thus appear not to yield bile pigment by the action of acids, but to be a precursor of bile pigment or of a bile pigment-haemoglobin yielding bile pigment on treatment with acids. While such an assumption would solve the difficult problem: what is the constitutional difference between cholehaem and verdohaem? it would create new vexing problems. If the bile pigments, which are obtained from choleglobin solutions by the action of acids, are not the iron-free prosthetic group of choleglobin, what can this prosthetic group be? Apart from bile pigments and protohaemin no other compounds were isolated from choleglobin solutions treated by acids. Protohaemin is exclusively derived from accompanying haemoglobin, since it decreases exactly in proportion to the haemoglobin concentration (cf. Part 3). We have also shown in Part 4 that the greater part of the choleglobin iron is removed by incubation with *N*/10 HCl for 16 hr.

A closer examination of the data in Table 8 reveals, indeed, sufficient evidence that choleglobin is split to bile pigments with a yield of 11–12 %. Such a yield is found in the initial periods in which according to Table 3 of Part 3 (p. 344) choleglobin is the only product of the reduction. This is evident from Table 9 in which the yield of unknown by-product obtained from Table 3 of Part 3, i.e. initial haemoglobin minus (choleglobin + haemoglobin + cholehaemochromogen + protohaemochromogen), is given in the third column and compared with the percentage yield of bile pigment in the fourth column. The same yield of 11–12 % is obtained from choleglobin produced by coupled oxidation of haemoglobin with glutathione or with thiolacetic acid. With the latter again no formation of by-products was indicated by the balance sheet of the reaction. The apparent bile pigment recovery from haemoglobin is somewhat higher, but still below 20 %, where there is a slight formation of by-product (under 3 % of the initial haemoglobin), e.g. in experiments with ascorbic acid at *p*H 8·5 up to 80 min. and at *p*H 7·2 up to 30 min. incubation time. Only where the balance sheet indicates a

Table 8. *Bile pigment yields from choleglobin solutions*

Haemoglobin 0·72%, ascorbic acid 0·1%, 37°

*p*H	Incubation time	Choleglobin in % of initial haemoglobin	μg. biliverdin from 50 ml.	μg. total bile pigment from 50 ml.	Biliverdin in % of theory	Total bile pigment in % of theory	Method	Remarks
7·2	15 min.	8·2	58	—	5·6	—	I	
		7·6	102	—	10·6 (7·3)	—	I	
		5·9	42	—	5·6	—	I	
		8·2	52	112	5·1	10·9	II	
		7·6	101	170	10·5 (7·8)	16·1 (13·5)	II	
	20 min.	11·2	127	217	9·0	15·4	II	
	30 min.	18·3	123	—	5·3	—	I	
		14·2	173	—	9·7 (8·4)	—	I	
		10·8	138	—	10·1	—	I	
		13·5	121	327	7·4	20·0	II	
		13·5	138	352	8·5 (8·6)	21·6 (19·3)	II	
		18·3	230	377	10·0	16·4	II	
	80 min.	[20]	360	1050	14·9	43·5	II	Precipitate; [ ]=not measured, average value of choleglobin
		—	393	990	16·3 (17·2)	41·0 (34·2)	II	
		—	520	753	20·6	29·8	II	
		—	427	562	16·9	22·5	II	
	2 hr.	[20]	914	1219	36·2	48·3	II	Precipitate
	5 hr.	[20]	1230	1500	48·7	59·5	II	Precipitate
	6½ hr.	[20]	1480	1690	58·7	67·0	II	Precipitate
	16 hr.	11·5	1030	—	71	—	I	Precipitate
8·5	15 min.	14·8	95	—	5·1	—	I	
		21·7	266	—	9·7 (6·5)	—	I	
		9·9	58	—	4·7	—	I	
		21·7	178	388	6·5	14·2	II	
		21·0	154	239	5·8 (5·6)	9·0 (10·1)	II	
		21·0	118	188	4·5	7·1	II	

	30 min.	32·6	482	—	11·7	—	I	
		32·1	465	—	11·5 (9·4)	—	I	
		21·0	194	—	7·3	—	I	
		31·9	279	—	7·2	—	I	
		32	242	360	6·0	8·9	II	
		32	322	560	8·0	13·9	II	
		32	180	268	4·5 (8·5)	6·7 (12·4)	II	
		37	465	810	10·0	17·3	II	
		24	209	350	7·0	11·7	II	
		31	464	616	11·9	15·8	II	
	50 min.	28·7	336	484	9·3	13·4	II	Precipitate
	80 min.	[28]	595	—	16·9	—	I	Precipitate; if filtrate alone split: 468 μg. = 13·3% biliverdin
		28	690	893	19·5	25·3	II	
		28	379	553	10·7 (14·9)	15·7 (19·1)	II	
		28	445	572	12·6	16·2	II	
	2 hr.	36·5	756	—	16·7 (26·6)	—	I	Precipitate
		21·4	990	—	36·6	—	I	
		21·0	681	857	25·8	32·5	II	
		[28]	1005	1225	28·5 (27·2)	34·7 (33·6)	II	
	3 hr.	[20]	1060	—	42·7 (41·0)	—	I	Precipitate
		—	990	—	39·3	—	I	
	4 hr.	[20]	1340	—	53	—	I	Precipitate; if filtrate alone split: 1170 μg. = 43% biliverdin
8·5	5 hr.	[20]	1505 (*a*)	—	60 (*a*)	—	I	(*a*) With precipitate. From filtrate alone: 784 μg. = 31%. (*b*) From filtrate alone
		—	910 (*b*)	—	36 (*b*)	—	I	
	6½ hr.	[20]	1030	—	41	—	I	From filtrate alone
	7½ hr.	[20]	1060	1215	42	48·3	II	From filtrate only
		—	1190	—	47	—	I	
	8½ hr.	[20]	1010	—	40	—	I	From filtrate only
	9½ hr.	[20]	775	—	31	—	I	From filtrate only
	16 hr.	13·6	620	—	36	—	I	From filtrate only
					Thiolacetic acid			
7·2	90 min.	19·5	220	307	8·5	11·9	II	Ascorbic acid added before acidification
					Glutathione			
7·2	19 hr.	6·0	50	86	6·6	11·4	II	Ascorbic acid added before acidification

Table 9. *Correlation between by-product formation during coupled oxidation and excess bile pigment not derived from choleglobin*

*p*H	Time of incubation min.	By-product formed in % of choleglobin equivalents	Average yield of bile pigment in % of choleglobin equivalents	Average yield of excess of bile pigment over 11·5 % in equivalent % of by-product
8·5 with ascorbic acid	15	0	10·1	—
	30	0	12·4	—
	80	2·6	19·1	82
	120	8·1	33·6	73
	420	25·9	48·3	28
7·2 with ascorbic acid	15	0·4	13·5	38
	30	2·8	19·3	41
	80	9·5	34·2	48
	120	12·4	48·3	59
	420	11·4	67·0	97
7·2 with thiol-acetic acid	90	0	11·9	—

considerable formation of by-product in the later stages of the reaction, much more bile pigment is obtained. It can also be seen from Table 9 that the initial rate of bile pigment formation at *p*H 8·5 is more than double that at *p*H 7·2, exactly as in the case with the rate of choleglobin formation. From these observations we conclude that choleglobin is split by 66 % acetic acid to 11·5 %, i.e. to about the same degree as verdoglobin (cf. Table 6). This also supports the view that the two substances are closely related to each other.

We have attempted to gain information on the question to what degree choleglobin is split by the treatment with 66 % acetic acid in other ways, but have failed to obtain a definite result. The unsplit choleglobin was precipitated from the aqueous solution remaining after ether extraction of bile pigments and protohaematin by sodium acetate and ammonium sulphate. It was then filtered off, washed free from salts and redissolved in *N*/10 NaOH. The absorption maximum at 630 mμ of the CO-cholehaemochromogen was measured and its strength indicated that about 70 % of the choleglobin (in one experiment 45 %) had been split. The colour of the recovered cholehaemochromogen is, however, rather brown than green, and it is likely that it has become altered and that its absorption has been diminished in intensity. The values found, give therefore, only minimum values of recovery of unsplit choleglobin. In the case of cholehaemochromogen smaller salt concentrations are required for the precipitation of the pigment and the estimation of recovery is more likely to be correct. While incubation with *N*/10 HCl removed 66 % of the iron from green pigment 83 % were recovered unsplit after 15 min. standing at room temperature in 60 % acetic acid.

The later part of the reaction with ascorbic acid is complicated by several factors. First, 'green pigment' precipitates and while little bile pigment is obtained from 'green pigment' precipitated after 80 min. incubation, later on a larger part of the bile pigment may be derived from precipitate (cf. the values of biliverdin obtained from the solution alone (last column) with those obtained from the unfiltered suspension (column 6 of Table 8). This bile pigment from green pigment may either be derived from a cholehaemochromogen, in which the protein has been denatured in such a way that no firm linkage between prosthetic group and protein is established, or, more likely, from an iron-free bile pigment-protein compound. In Part 3 (p. 342) we have indeed reported evidence for the

occurrence of some iron-free protein in the 'green pigment'. On longer incubation ascorbic acid evidently removes iron from cholehaemochromogen. The figures in the last column of Table 8 show, however, that the bile pigment recoveries from the filtrate alone also still far exceed the 11·5% derived from choleglobin. In the last column of Table 9 the bile pigment derived from choleglobin, assuming a yield of 11·5%, is subtracted from bile pigment found, and this excess is given in equivalent percentage of the unknown by-product. This percentage cannot be established with great accuracy, but the figures leave no doubt that the by-product yields a much larger amount of bile pigment than choleglobin or verdoglobin. It is therefore likely that prolonged incubation of haemoglobin with ascorbic acid sets free bile pigment from choleglobin, and that this occurs now in the solution still bound to protein, but no longer to iron. This excess bile pigment not derived from choleglobin consists almost entirely of biliverdin. Since biliverdin is reduced by $Na_2S_2O_4$ to a yellow compound with no absorption in the red and green parts of the spectrum, the spectrophotometric determination of the cholehaem and protohaem compounds is not affected by its presence, and in the balances of Table 3, Part 3 the by-product appears simply as deficit.

Additions of further ascorbic acid before the acid splitting of choleglobin or of CO do not alter the total yield of bile pigment; they increase the yield of biliverdin slightly and decrease the yield of biliviolin correspondingly.

Isolation of bile pigments from red cells

The bile pigment yield from red cells is small and for its estimation rather strong solutions of haemoglobin must be employed. It is to be expected that the recovery of bile pigment from such solutions will be less complete than from dilute haemoglobin solutions. On the other hand the yield of bile pigment obtained from the blood or from strong solutions of haemoglobin can no longer be neglected in the estimation of the recovery of added bile pigment. We therefore measured the amount of bile pigment that can be isolated from red cells in one sample and determined the degree of recovery of added biliverdin in a second sample of the same red cell solution. In order to avoid oxidation of the biliverdin, ascorbic acid (50 mg. per 100 ml.) was added immediately before acidification with acetic acid.

20 ml. of fresh sheep cells, twice washed with saline were diluted to 100 ml. with water and haemolysed. 50 ml. of this solution were treated with ascorbic acid and acetic acid, while to the second lot of 50 ml. a solution of 222 μg. biliverdin in 1 ml. dilute ammonia was added before they were treated in the same way. Both samples were worked up in the manner described with a few modifications. With the high protein concentrations great care had to be taken to avoid the formation of emulsions. The greater part of the protein was precipitated by the addition of somewhat more ether than was used for the recovery of bile pigment from dilute solution, and filtered off before the addition of sodium acetate solution and water. It was impossible to avoid precipitation of some haemin from the ether before complete removal of the protein; this was filtered off from the ethereal solution, or if there was not so much as to disturb the separation of the layers, from the acid extracts, which had to be filtered in any case. Both samples were worked up in precisely the same manner. 62 μg. bile pigment (42 μg. biliverdin and 16 μg. biliviolin) were isolated from the first sample and 162 μg. biliverdin from the second. From the added 222 μg. thus only $162-46=116$ μg. $=52\%$ were recovered.

If it is assumed that the same percentage of the bile pigment set free by splitting the bile pigment-haemoglobin of the red cells is recovered as from added biliverdin, and that the bile pigment-haemoglobin of the red cells is split to the same degree as choleglobin or verdoglobin, the amount of bile pigment found has to be multiplied by the factor $\frac{100}{52} \cdot \frac{100}{11{\cdot}5} \cdot \frac{16{,}700}{584} = 480$ in order to obtain the amount of bile pigment-haemoglobin, from which it was derived. 62 μg. bile pigment thus correspond to 29·8 mg. bile pigment-haemoglobin. Since the 10 ml. of laked cells contained 3270 mg. haemoglobin, only 0·9 % of the red cell haemoglobin was bile pigment-haemoglobin.

In a second sample 8·75 ml. of sheep erythrocytes containing 2·8 g. haemoglobin yielded 66·2 μg. bile pigment (45·7 μg. biliverdin and 20·5 μg. biliviolin) without the addition of ascorbic acid, while with ascorbic acid only 46 μg. (23 μg. biliviolin and 23 μg. biliverdin) were obtained. These values correspond to bile pigment contents of 1·1 and 0·8 %.

Horse erythrocytes were not studied quantitatively, but qualitatively they gave the same results. Similar yields to those from sheep red cells were obtained from solutions of crystalline horse oxyhaemoglobin. 50 ml. of a 5·7 % solution gave 76 μg. bile pigment (39 μg. verdin and 37 μg. violin), corresponding to a bile pigment-haemoglobin content of 1·3 %, when CO-haemoglobin was split in the presence of 50 mg. ascorbic acid, while the splitting of the oxyhaemoglobin without ascorbic acid gave here a smaller yield of 35 μg. (of which 30 μg. were violin) corresponding to 0·6 % bile pigment-haemoglobin.

These estimations are not very accurate, but while proving the presence of some bile pigment-haemoglobin in red cells, they make it unlikely that bile pigment-haemoglobin constitutes more than a part of the 'labile iron' of the blood. Since splitting in the presence of ascorbic acid and of CO decreases the yield of bile pigment little, if at all, the latter cannot be derived to a large extent from oxidative processes occurring during the acidification of oxyhaemoglobin solutions, but must be derived, at least partly, from bile pigment-haemoglobin preformed in the red cells. The biliviolin obtained appeared to be of the weakly basic type III, but not sufficient material was available to identify it with the biliviolin III*a* obtained from choleglobin.

Summary

On splitting with acetic acid choleglobin yields biliverdin and a weakly basic biliviolin (biliviolin III*a*). Cholehaemochromogen ('green pigment') gives small amounts of the same products, but the greater part of the prosthetic group has become firmly attached to the denatured protein. On splitting with ferrous acetate-acetic acid-hydrochloric acid green pigment yields small amounts of a hydroxyporphyrin. Verdohaemochromogen is split by acids to biliverdin and a biliviolin of medium basicity (biliviolin II*b*).

Quantitative methods are worked out allowing the isolation of bile pigments from solutions that contain oxyhaemoglobin, and for their spectrophotometric and colorimetric determination. An average recovery of 85 % is obtained. Biliverdin is oxidized during the action of acids on oxyhaemoglobin to biliviolin II*b*, and this oxidation is prevented by ascorbic acid.

Verdohaematin is split to bile pigments by 66 % acetic acid to an extent of 25–35 %, verdoglobin to 10–15 %. The bile pigment yield from choleglobin is 11–12 % in the initial phases of the coupled oxidation of haemoglobin and ascorbic acid, in which no by-product is formed. Later considerably more bile pigment is obtained. This bile pigment, not derived from choleglobin, is biliverdin

and the by-product is probably an iron-free biliverdin-protein compound. Prolonged action of ascorbic acid on choleglobin is thus able to remove the iron held in complex combination in choleglobin and to set free biliverdin at a physiological *p*H.

Small amounts of bile pigments (biliverdin and biliviolin) were obtained by treatment with acetic acid from laked red cells of sheep and horse as well as from crystalline horse oxyhaemoglobin. Provided that the bile pigment haemoglobin, from which these bile pigments are derived, is split to the same degree as choleglobin, and that the percentage of recovery of these bile pigments is the same as that of added biliverdin (52 %), only about 1 % of the red cell iron is bile pigment-haemoglobin iron.

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37. PYRUVIC DEHYDROGENASE OF *BACTERIUM COLI*

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Bacterium coli oxidizes pyruvate to CO_2 and water [Cook & Stephenson, 1928]; and in the presence of dinitrophenol or NaN_3 the oxidation is complete [Clifton & Logan, 1939]. Anaerobically, the main products of the breakdown of pyruvate are acetic, lactic, formic and succinic acids, ethyl alcohol, H_2 and CO_2 [Tikka, 1935]. The theories concerning the oxidation have been summarized by Mazza & Cimmine [1934]. More recent work appeared in two papers by Krebs [1937, 1, 2].

Cell-free extracts of *Bact. coli* prepared by crushing the cells in the Booth-Green mill were found to contain an enzyme system capable of oxidizing pyruvate. This paper reports the study of some of the properties of such extracts.

I. *Methods*

Pyruvic acid was estimated by the bisulphite fixation method of Clift & Cook [1932]. The cocarboxylase was prepared by Dr H. Weil-Malherbe, to whom the author is grateful.

The activity of the enzyme system was followed by measuring the O_2 uptake in Warburg manometers at 37°. Anaerobically, methylene blue acts as hydrogen acceptor for the reaction.

II. *Preparation of the extracts*

Bacterium coli-commune Escherich (National Collection of Type Cultures No. 86) was grown on agar with tryptic digest of casein, lactate and yeast water. Yeast water was added to ensure an adequate supply of substances required for synthesis of cocarboxylase [Silverman & Werkman, 1939]. After 24–30 hr. growth the cells were washed off, centrifuged and crushed for 2 hr. in the wet-crushing mill [Booth & Green, 1938]. The crushed cells were diluted with an equal volume of distilled water and centrifuged at 12,000 r.p.m. for 40 min. The clear yellow supernatant fluid was dialysed for 14 hr. against running water.

III. *Carboxylase*

The presence of carboxylase in the fresh undialysed extracts could not be shown under aerobic conditions. Similar extracts of crushed yeast cells showed high carboxylase activity under the same conditions. This confirms the conclusion of Cook [1930] that pyruvate is not decarboxylated by *Bact. coli* in the same way as by yeast.

IV. *Components of the system*

Lipmann [1939, 3 for summary] has obtained from *Lactobacillus delbrückii* (an anaerobe which does not oxidize acetate) phosphate extracts which oxidize pyruvate to acetate and CO_2. The oxidizing system comprises at least the following: (1) a protein or proteins, (2) inorganic phosphate, (3) cocarboxylase, (4) a metal, Mg, Mn or Co and (5) flavin adenine dinucleotide.

The dialysed extracts of *Bact. coli* resemble Lipmann's preparations in several respects, as indicated in Table 1.

Table 1. *Components of the oxidizing system*

	Additions to manometers (ml.)				
Extract	1·0	1·0	1·0	1·0	1·0
M/30 phosphate *p*H 6·17	—	0·9	0·9	0·9	0·9
Cocarboxylase 100 μg./ml.	0·4	0·4	0·4	—	0·4
Manganese 100 μg./ml.	0·3	0·3	0·3	0·3	—
M pyruvate	0·2	0·2	—	0·2	0·2
Water	1·3	0·4	0·6	0·8	0·7
O_2 uptake, μl./60 min.	49·5	235·0	17·5	32·0	143·0

Phosphate, cocarboxylase and a metal are apparently involved in the oxidation of pyruvate by the dialysed extract. The degree of dependence upon added phosphate varied greatly in different preparations. Flavin adenine dinucleotide caused a small increase in the rate of oxidation. It was possible to treat the extract with ammonium sulphate up to 62 % of saturation and retain the active system in the precipitate, most of which dissolved in glass-distilled water to give an orange-yellow solution. Such treatment removed substrate, cocarboxylase and much of the metal component (Table 2).

Table 2. *Components of the precipitated system after dialysis*

	Additions to manometers (ml.)				
Enzyme	1·0	1·0	1·0	1·0	1·0
M/15 phosphate *p*H 6·17	—	1·5	1·5	1·5	1·5
Cocarboxylase 100 μg./ml.	0·2	0·2	0·2	—	0·2
Manganese 100 μg./ml.	0·3	0·3	0·3	0·3	—
M pyruvate	0·2	0·2	—	0·2	0·2
Water	1·5	—	0·2	0·2	0·2
O_2 uptake, μl./45 min.	141·0	138·5	0	3·5	39·0

(1) *Protein.* By fractional precipitation of the extract it was found that the precipitate obtained by the addition of ammonium sulphate up to 40 % saturation contained very little activity. This fraction was not completely soluble when re-suspended in water and dialysed. It contained a very active lactic dehydrogenase. The precipitate obtained between 40 and 62 % saturation with ammonium sulphate contained most of the pyruvic enzyme. This fraction re-dissolved in glass-distilled water to give a greenish yellow solution which became slightly opalescent after dialysis. This fraction also contained some of the lactic dehydrogenase. This separation was not always complete with every preparation. However, it was observed that while the 40–62 % fraction was always more active, its activity could be greatly increased by the addition of some of the 0–40 % fraction. The combined activity was considerably more than the activity of the two fractions separately (Table 3). The factor present in the 0–40 % fraction responsible for this phenomenon was non-dialysable and heat-labile. In one instance both fractions were inactive before mixing.

As it seems likely that this system is similar to that of *Lactobac. delbrückii*, the effect may be due to the partial separation of two enzymes. The reaction, as Lipmann pictures it, involves two coenzymes or prosthetic groups which have so far been considered as owing their biological activity to their union with specific proteins. In the case of the pyruvic dehydrogenase, the question arises whether cocarboxylase and flavin adenine dinucleotide have a specific protein

Table 3. *Activities of the fractions*

	Additions to manometers (ml.)						
Fraction 0–40%	0·5	0	0·5	0·5	0·5	0·5	0·5
Fraction 40–62%	—	1·0	1·0	1·0	1·0	1·0	1·0
M/20 phosphate *p*H 6·17	1·0	1·0	1·0	1·0	1·0	—	1·0
M pyruvate	0·2	0·2	0·2	0·2	0·2	0·2	—
Cocarboxylase 100 μg./ml.	0·2	0·2	0·2	0·2	—	0·2	0·2
Manganese 100 μg./ml.	0·3	0·3	0·3	—	0·3	0·3	0·3
Water	1·0	0·5	—	0·3	0·2	1·0	0·2
O_2 uptake, μl./40 min.	14·5	172·0	353·0	52·5	9·0	370·0	8·0

each, or one protein for the two groups. If there are two, the accelerating effect obtained after mixing the two fractions may be due to the fact that one of the proteins is almost completely absent from one of the fractions.

(2) *Inorganic phosphate*. The necessity for this component of the system could be demonstrated for certain only with enzyme extract after dialysis and before precipitation with ammonium sulphate (Table 1). Ammonium sulphate of B.D.H. A.R. standard was used throughout. Attempts to obtain preparations showing no oxidation unless phosphate was added were unsuccessful. Extensive dialysis inactivates the enzyme; so also does heating to 55–60°, which was a useful method for removing inactive protein from bacterial extracts containing soluble dehydrogenases [Still, 1940, 1].

Arsenate was found to replace phosphate in facilitating the oxidation of pyruvate by the dialysed extract (Table 4). This recalls the fact that arsenate will replace the phosphate required for the oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid [Still, 1940, 2].

Table 4. *Effect of arsenate and phosphate*

Manometers contained 1·0 ml. dialysed extract, 20 μg. cocarboxylase, 30 μg. Mn and 0·2 ml. *M* pyruvate. Total volume 3·2 ml.

	O_2 uptake μl./60 min.
No phosphate	26
0·4 ml. *M*/5 phosphate	121
0·4 ml. *M*/5 arsenate	107

Lipmann has shown that when pyruvate is oxidized by dried cells of *Lactobac. delbrückii* in the presence of adenylic acid, there is a simultaneous phosphorylation of the adenylic acid. Depending largely upon the amount of adenylpyrophosphatase present in the cells and its degree of inhibition by NaF, the accumulation of adenylpyrophosphate could be demonstrated in the reaction mixture [Lipmann, 1939, 1].

Using the dialysed extract of crushed cells of *Bact. coli* no conclusive evidence could be adduced for the accumulation of adenylpyrophosphate, even in presence of *M*/32 NaF (Table 5). It was found in these experiments that the incubation of the extract with adenylic acid caused a considerable increase in inorganic phosphate whether or not pyruvate had been oxidized at the same time. In the absence of adenylic acid, incubation of the extract with or without pyruvate produced only a small increase in inorganic phosphate. *Bact. coli* rapidly dephosphorylates and then deaminates adenylic acid [Lutwak-Mann, 1936]. Both NaF and adenylic acid caused some inhibition of the rate of oxidation of pyruvate.

After precipitation with ammonium sulphate, the O_2 uptake of the fraction precipitated between 40 and 62% saturation was considerably increased by the

Table 5. *Oxidation and phosphorylation by dialysed extract*

		Additions to manometers (ml.)			
Extract		1·0	1·0	1·0	1·0
M/5 phosphate *p*H 6·17		0·3	0·3	0·3	0·3
Cocarboxylase 100 μg./ml.		0·4	0·4	0·4	0·4
Manganese 100 μg./ml.		0·3	0·3	0·3	0·3
M pyruvate		—	0·4	0·4	—
M NaF		0·1	0·1	0·1	0·1
0·25 *M* adenylic acid		—	—	0·3	0·3
Water		0·7	0·3	—	0·4
O_2 uptake, μl./90 min.		15·0	563·0	426·0	27·5
	Initial				
Inorganic P, mg.	1·81	2·02	1·90	3·02	3·07
Hydrolysable P, mg.	0·05	0·01	0	0	0·06

Phosphate removed by 7 minutes' boiling with *N* HCl was considered as having been derived from adenylpyrophosphate.

addition of inorganic phosphate (Table 6). Using this fraction it was possible to show the formation of adenylpyrophosphate during the oxidation of pyruvate (Table 7). Presumably the adenylpyrophosphatase was largely removed or inactivated during fractionation with ammonium sulphate. The oxidation of pyruvate by this fraction was not inhibited by NaF (Table 6).

Table 6. *Components of the active fraction*

	Additions to manometers (ml.)					
Fraction 40–62%	1·0	1·0	1·0	1·0	1·0	1·0
M/5 phosphate *p*H 6·17	0·5	—	0·5	0·5	0·5	0·5
Cocarboxylase 100 μg./ml.	0·4	0·4	0·4	—	0·4	0·4
Manganese 100 μg./ml.	0·3	0·3	0·3	0·3	—	0·3
M NaF	0·1	0·1	0·1	0·1	0·1	—
0·8 *M* pyruvate	0·5	0·5	—	0·5	0·5	0·5
Water	0·4	0·9	0·9	0·8	0·7	0·5
O_2 uptake, μl./60 min.	145·0	37·0	0	11·0	16·0	156·0

Table 7. *Oxidation and phosphorylation by active fraction*

		Additions to manometers (ml.)			
Fraction 40–62%		1·0	1·0	1·0	1·0
M/5 phosphate *p*H 6·17		0·3	0·3	0·3	—
Cocarboxylase 100 μg./ml.		0·4	0·4	0·4	0·4
Manganese 100 μg./ml.		0·3	0·3	0·3	0·3
M NaF		0·1	0·1	0·1	0·1
0·8 *M* pyruvate		0·5	0·5	—	0·5
0·25 *M* adenylic acid		—	0·6	0·6	—
Water		0·6	—	0·5	0·9
O_2 uptake, μl./160 min.		340·0	489·0	0	87·5
	Initial				
Inorganic P, mg.	2·08	2·10	2·59	2·75	0·34
Hydrolysable P, mg.	0	0	0·14	0	0

(3) *Cocarboxylase.* Table 8 shows the response of the dialysed extract to the addition of cocarboxylase. No special precautions were taken to incubate the enzyme and coenzyme together before the reaction for any time longer than the equilibrium period of the manometers.

Tables 1, 2 and 3 show that most of the cocarboxylase can be removed by dialysis and practically all of it by precipitation of the enzyme with ammonium

Table 8. *Effect of cocarboxylase on oxidation of pyruvate*

Manometers contained 1·0 ml. dialysed extract, 1·6 ml. *M*/100 phosphate *p*H 6·17, and 0·2 ml. *M* pyruvate. Total volume 3·2 ml.

Cocarboxylase μg./3·2 ml.	μl. O_2/41 min.
—	10
2·5	86
5·0	143
7·5	171
10·0	181
15·0	184
20·0	191

sulphate and subsequent dialysis. The fact that reactivation of the enzyme by recombination with cocarboxylase takes place after dialysis indicates a point of difference between this enzyme and carboxylase. Green *et al.* [1940] have shown that once cocarboxylase has been dialysed from carboxylase, subsequent addition of cocarboxylase does not restore the lost activity.

(4) *Metal component.* Addition of Mn, Mg or Co to the dialysed extract increased the rate of oxidation of pyruvate in the presence of cocarboxylase. The increase was more marked if the enzyme had been precipitated with ammonium sulphate and dialysed, or incubated for some time and dialysed again (Table 9). Precipitation with ammonium sulphate at an acid reaction was found to be necessary in order to remove the metal component from the pyruvic dehydrogenase system of *Lactobac. delbrückii* [Lipmann, 1939, 3]. Tables 1, 2, 3 and 6 show the partial removal of this component when the extracts are precipitated.

Table 9. *Effect of metals*

The dialysed extract was incubated at 37° for 45 min., diluted once and dialysed once again for 2 hr. against distilled water. Manometers contained 1·0 ml. enzyme, 0·5 ml. *M*/5 phosphate, 0·2 ml. *M* pyruvate and 20 μg. cocarboxylase. Total volume 3·2 ml.

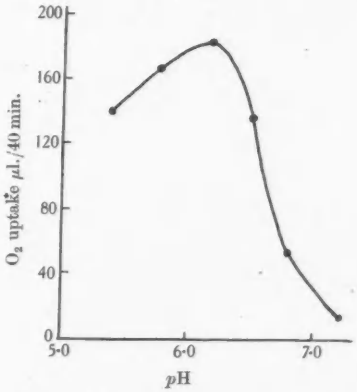
	μl. O_2/30 min.
No addition	0
Manganese, 30 μg.	64·5
Magnesium, 30 μg.	60·0
Cobalt, 30 μg.	15·0

(5) *Flavin component.* Addition of flavin adenine dinucleotide (F-A-D) to the dialysed extract increased the rate of oxidation of pyruvate by about 30 %. In view of the participation of F-A-D in the oxidation of pyruvate by extracts of *Lactobac. delbrückii* [Lipmann, 1939, 2], attempts were made to split reversibly the enzyme from *Bact. coli*. The dialysed extract was half saturated with ammonium sulphate at *p*H 3·0 and at 0°. Little of the resulting precipitate was soluble when re-suspended in water. The suspension, when tested under the standard conditions, did not oxidize pyruvate in the presence or absence of F-A-D. While the above procedure was followed by Lipmann [1939, 2] for phosphate extracts of *Lactobac. delbrückii*, less drastic treatment would seem to be more suitable for the extracts of *Bact. coli*.

V. *Reaction with molecular oxygen*

pH. The optimum *p*H for the oxidation of pyruvate by the dialysed extract is about 6·2. Fig. 1 shows the relation between *p*H and the rate of O_2 uptake in *M*/8·9 phosphate. Actually the O_2 uptake is quite appreciable in the absence

of added buffer. Substitution of citrate or acetate buffer markedly inhibits the reaction. This effect of citrate may be due to its combination with the metal component.



F.g. 1. Effect of *p*H on oxidation of pyruvate. Warburg manometers contained 1·0 ml. dialysed extract, 0·2 ml. cocarboxylase 100 μg./ml., 0·2 ml. *M* pyruvate, 1·8 ml. *M*/5 phosphate buffer. Total volume 3·2 ml.

Substrate. A considerable concentration of pyruvate is required to saturate the enzyme (Table 10), and it was found that pyruvate remained in the reaction mixture even after 3–4 hr. incubation.

Table 10. *Effect of concentration of pyruvate*

Manometers contained 1·0 ml. dialysed extract, 1·6 ml. *M*/15 phosphate *p*H 6·17, 20 μg. cocarboxylase. Total volume 3·2 ml.

Concentration of pyruvate m-Mol./l.	μl. O_2/25 min.
—	0
3·0	24·5
6·0	37·5
12·0	62·5
25·0	85·0
62·0	122·5
125·0	119·5

Phenyl pyruvate, at a concentration of *M*/80, was not oxidized under the same conditions.

Oxygen equivalent. The O_2 uptake per molecule of pyruvate oxidized is the equivalent of one atom of oxygen per molecule of pyruvate (Fig. 2). This result corresponds to the reaction (1):

$$CH_3.CO.COOH + \tfrac{1}{2}O_2 = CH_3.COOH + CO_2. \qquad (1)$$

Fig. 3 shows the O_2 uptake and CO_2 output during the oxidation of pyruvate, measured simultaneously by the indirect method of Warburg. As the residual pyruvate was not determined in this instance, the percentage of theoretical O_2 uptake and CO_2 output was greater than is indicated in the figure. It will be seen that the average R.Q. over 2 hr. was 0·45 against 0·50 which would be

required by (1). Fig. 3 also shows the anaerobic CO_2 output over the same period. This did not reach one-quarter of the aerobic CO_2 output, which fact would seem to eliminate the possibility that pyruvate is not oxidized directly but is first dismuted according to (2):

$$2CH_3.CO.COOH = CH_3.CHOH.COOH + CH_3.COOH + CO_2, \qquad (2)$$

and that the subsequent O_2 uptake is due to direct oxidation of lactate.

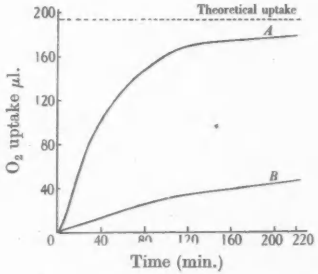


Fig. 2.

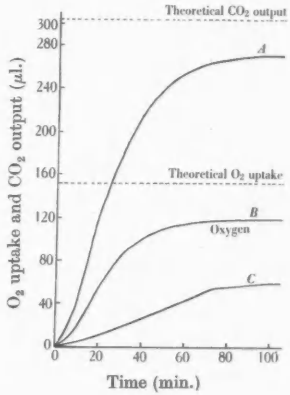


Fig. 3.

Fig. 2. Oxidation of pyruvate (*A*) and acetaldehyde (*B*). *A*, Warburg manometers contained 1·5 ml. enzyme (40–62% fraction), 0·7 ml. *M*/5 phosphate buffer *p*H 6·17, 0·4 ml. cocarboxylase 100 μg./ml., 0·3 ml. Mn 100 μg./ml., 0·3 ml. 0·07 *M* pyruvate. Total volume 3·2 ml. Blank without substrate subtracted. *B*, as above but containing 0·3 ml. *M* acetaldehyde in place of pyruvate.

Fig. 3. Oxidation and dismutation (*C*) of pyruvate. Warburg manometers contained 1·7 ml. enzyme (40–62% fraction), 0·6 ml. *M*/5 phosphate buffer *p*H 6·17, 0·4 ml. cocarboxylase 100 μg./ml., 0·3 ml. Mn 100 μg./ml., 0·2 ml. 0·07 *M* pyruvate. Total volume 3·2 ml. *A*, CO_2 output. *B*, O_2 uptake. *C*, CO_2 output. In *C*, the gas space contained N_2 instead of air.

Krebs [1937, 2], using intact cells of *Bact. coli*, found that under neutral or slightly acid conditions pyruvate was broken down mainly according to (3):

$$CH_3.CO.COOH + H_2O = CH_3.COOH + HCOOH. \qquad (3)$$

This reaction had been suggested previously by Tikka [1935] to account for the acetic and formic acids produced in *Bact. coli* fermentation. Under more acid conditions and in the presence of 1 atmosphere of CO_2, the process approximated more closely to (2), which Krebs found applicable to the breakdown of pyruvate by cocci and animal tissues. Barron & Lyman [1939] discuss these views in so far as they relate to certain cocci.

VI. *Product of oxidation*

Acetic acid was detected by its reaction with lanthanum nitrate [Krüger & Tschirch, 1930]. The absence of propionic acid was considered a reasonable assumption under the conditions of the experiments.

Acetic acid was estimated by the method of Friedemann [1938] owing to the presence of residual pyruvate which is to some extent volatile in steam. The following were incubated for 4 hr. in a Krebs vessel, O_2 being bubbled through

the mixture: 12 ml. enzyme solution (40–62 % fraction of the extract), 600 μg. cocarboxylase, 400 μg. Mn, 8 ml. *M*/5 phosphate *p*H 6·17 and 59·8 mg. pyruvic acid brought to *p*H 6·17, the total volume being 22 ml. After the incubation the mixture was made up to 25 ml. and the residual pyruvate and the acetic acid formed were estimated. Two distillations were made; one, a steam distillation in the presence of tungstate and $MgSO_4$ and then a redistillation in the presence of tungstate, $MgSO_4$ and $HgSO_4$. This procedure was followed in order to exclude lactic, pyruvic or formic acid from the final titration. A satisfactory recovery of the acid was obtained; thus, 24·94 mg. pyruvic acid disappeared and 17·38 mg. acetic acid were recovered. The amount calculated from reaction (1) would be 17·0 mg.

If similar experiments were carried out and the reaction mixture was deproteinized by heating with dilute H_2SO_4, residual pyruvic acid could be demonstrated as the 2:4-dinitrophenylhydrazone. In addition the mixture contained a substance giving a 2:4-dinitrophenylhydrazine derivative insoluble in $NaHCO_3$, and presumably not acidic. This question has not been further investigated, but the matter recalls the observations of Quastel & Wooldridge [1929] on the products of fermentation of pyruvate by *Bact. coli*.

VII. *Oxidation of acetaldehyde*

The extract oxidized acetaldehyde under the same conditions as it oxidized pyruvate; i.e. without the addition of coenzyme I or methylene blue. However, as will be seen in Fig. 2, the rate of oxidation was small. This does not support the view that acetaldehyde as such is an intermediate in the oxidation of pyruvate to acetate. During the oxidation of pyruvate it was not possible to detect the presence of acetaldehyde using Rimini's reaction [Bernhauer, 1936]. Acetate is not oxidized by the preparation. The ability to oxidize acetaldehyde disappears after heating to 55° [Still, 1940, 1].

VIII. *Anaerobic breakdown of pyruvate*

Anaerobically, the intact cells liberated CO_2 from pyruvate, oxaloacetate and acetoacetate, but not from mesoxalate or α-ketoglutarate. These experiments were carried out in N_2 and phosphate buffer at *p*H 6·0. In $NaHCO_3$-CO_2 buffer pyruvate was also decomposed, the optimum *p*H being about 7·2. The anaerobic reaction has not been sufficiently studied to justify any statement as to the relative importance of reactions (2) and (3) under these conditions.

Fresh undialysed extract of the crushed cells decomposed pyruvate in $NaHCO_3$-CO_2 buffer at *p*H 7·2 at a rate which was increased by the addition of cocarboxylase. The same extract also liberated CO_2 from pyruvate in N_2 and phosphate at *p*H 6·0, and the latter reaction could be increased by addition of cocarboxylase (Table 11). Adenylic acid and coenzyme I were without effect.

Table 11. *Effect of cocarboxylase on anaerobic decomposition*

Manometers contained 1·0 ml. undialysed extract, 0·2 ml. *M* pyruvate, 1·5 ml. *M*/15 phosphate *p*H 6·0. Total volume 3·2 ml. N_2 in the gas space.

	μl. CO_2/60 min.
Without pyruvate	4·5
With pyruvate	60·0
With pyruvate +20 μg. cocarboxylase	89·0

This anaerobic reaction under acid conditions was not marked, and after dialysis, or treatment with ammonium sulphate and subsequent dialysis, it was

considerably diminished. Activity was not restored by the addition of riboflavin, which has been found to be essential for the dismutation of pyruvate by air-dried cells or phosphate extracts of *Lactobac. delbrückii* anaerobically under acid conditions [Lipmann, 1939, 3]. This result suggests that the reaction occurring in the extract of *Bact. coli* is probably not dismutation by reaction (2). However, the absence of a dismutation mechanism in cells grown and maintained aerobically, may be quite normal.

IX. *Oxidation of lactate*

In view of the probability that dismutation of pyruvate is brought about by the system, pyruvic dehydrogenase—carrier—lactic dehydrogenase, the occurrence of a very active lactic dehydrogenase in the cell-free extracts of *Bact. coli* is significant. The enzyme used in the experiments of Table 12 was the fraction of the extract precipitated by 40 % saturation with ammonium sulphate and then dialysed. The results in Table 12 agree with those obtained by Stephenson [1928] working with a soluble lactic dehydrogenase prepared from autolysed cells of *Bact. coli*. The enzyme does not require coenzyme I, is not inhibited by the product of the oxidation and requires a carrier in order to react with molecular O_2. This enzyme seems to be identical with the lactic dehydrogenase of yeast recently obtained in a very pure state by Dixon & Zerfas [1939].

Table 12. *Oxidation of lactate*

	Additions to manometers (ml.)				
Enzyme, 0–40 % fraction	0·5	0·5	0·5	0·5	0·5
M/10 phosphate *p*H 7·3	1·5	1·5	1·5	1·5	1·5
2 *M* lactate	0·1	—	0·1	0·1	0·1
Coenzyme I (27 % pure) 10 mg./ml.	0·1	0·1	—	0·1	0·1
Methylene blue 0·5 %	0·5	0·5	0·5	—	0·5
2 *M* NaCN	0·2	0·2	0·2	0·2	—
Water	0·3	0·4	0·4	0·8	0·5
O_2 uptake, μl./21 min.	222·0	25·0	238·0	19·0	250·0

Discussion

From the results of this investigation it would seem probable that *Bact. coli* contains a pyruvic oxidizing system very similar to the pyruvic dehydrogenase of *Lactobac. delbrückii*. Until the enzyme is reversibly split or other evidence for the participation of flavin adenine dinucleotide in the oxidation is obtained, complete identity remains in doubt. Under anaerobic conditions, the 'hydroclastic' reaction (3) is probably much more important than dismutation (2), but this has not been fully investigated as yet.

No attempts have been made to isolate phosphorylated compounds intermediate between pyruvic acid and acetic acid. The removal of phosphorylated substances already in the enzyme preparation presents some difficulties. In this connexion it is interesting that Silverman & Werkman [1940] recently found that the conversion of pyruvic acid into acetyl methyl carbinol by extracts of *Aerobacter aerogenes* also required the presence of inorganic phosphate. Virtanen & Karström [1931] first observed that inorganic phosphate was required for the oxidation of pyruvic acid by bacteria.

SUMMARY

1. Cell-free extracts of *Bact. coli* do not contain carboxylase, but oxidize pyruvic acid to acetic acid and CO_2.

2. This pyruvic dehydrogenase system has been shown to comprise at least (*a*) a protein or proteins, (*b*) inorganic phosphate, (*c*) cocarboxylase and (*d*) a metal, possibly manganese or magnesium. Flavin adenine dinucleotide may also be a component of the system.

3. The oxidation of pyruvic acid in the presence of adenylic acid is probably coupled with the phosphorylation of the latter substance.

4. The extracts also decompose pyruvic acid under anaerobic conditions.

5. Acetaldehyde is oxidized only slowly, and could not be detected during the oxidation of pyruvic acid.

6. The extracts oxidize lactate rapidly in the presence of a carrier.

The author is grateful to Dr D. E. Green, Dr M. Stephenson and Dr R. P. Cook for their advice and interest in this work; and to Hebden Medical Fund of the University of Sydney for an assistance grant. The crushing mill used in this investigation was provided by a grant to this laboratory from the Medical Research Council.

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38. GLYOXALASE OF *BACTERIUM COLI*

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(*Received 31 January 1941*)

Neuberg & Gorr [1925] observed that *Bact. coli*, both in culture and when dried, caused the conversion of methylglyoxal into lactic acid. Using yeast and *B. pasteur*, Lohmann [1932] investigated the properties of glyoxalase, the enzyme concerned, and determined the nature of its coenzyme or prosthetic group.

Cell-free extracts of *Bact. coli* prepared with the wet-crushing mill of Booth & Green [1938] contained an active glyoxalase. The following results were obtained with such extracts.

I. *Preparation.* *Bacterium coli commune* Escherich (National Collection of Type Cultures No. 86) was the source of the enzyme. Extracts were prepared as previously described [Still, 1941]. The extracts were used without heating or fractional precipitation, and retained their activity for 10 days without appreciable loss if kept at 0°.

II. *Methods.* The activity of the enzyme was measured in Warburg manometers by following the liberation of CO_2 from $NaHCO_3$-CO_2 buffer at *p*H 7·2. The methylglyoxal was prepared from 'Oxantin' by distillation in the presence of H_2SO_4 and estimated by the bisulphite fixation method of Clift & Cook [1932].

III. *Coenzyme.* The extract as prepared above showed very little activity, and this could be almost completely elminated by dialysis against running water for 12 hr. (Table 1).

Table 1. *Effect of dialysis on glyoxalase in extract*

Manometers containing 1·0 ml. extract, 0·4 ml. *M*/10 $NaHCO_3$, 0·4 ml. 0·23 % methylglyoxal. Total volume 3·2 ml.

	μl. CO_2/31 min.
Extract undialysed, no substrate	1·5
Extract undialysed, + substrate	25·0
Extract dialysed, no substrate	4·5
Extract dialysed, + substrate	2·0
No enzyme, + substrate	2·0

Addition of reduced glutathione more than restored the activity present before dialysis (Table 2).

Table 2. *Effect of GSH on activity of extract*

Manometers containing 1·0 ml. extract, 0·4 ml. *M*/10 $NaHCO_3$, 0·4 ml. 0·47 % methylglyoxal and 3·0 mg. GSH. Total volume 3·2 ml.

	μl. CO_2/20 min.
Extract undialysed, no substrate	3·0
Extract undialysed, + substrate	190·0
Extract dialysed, no substrate	3·5
Extract dialysed, + substrate	143·5
Extract dialysed, + substrate, no GSH	6·0

Oxidized glutathione in equivalent amounts did not replace GSH as coenzyme. However, an equivalent amount of cysteine brought about a considerable

increase in the glyoxalase activity of the dialysed extract (Tables 3 and 4). Lohmann [1932] showed that with his yeast preparations no other SH-compound could replace GSH as coenzyme for glyoxalase. No explanation is offered for the action of cysteine in this instance.

Table 3. *Effect of concentration of GSH*

Manometers containing 1·0 ml. dialysed extract, 0·4 ml. *M*/10 $NaHCO_3$, 1·7 mg. phenylglyoxal. Total volume 3·2 ml.

GSH mg.	μl. CO_2/17 min.
—	26·0
0·25	60·5
0·5	70·5
1·0	85·0
2·0	96·0
3·0	88·0

Table 4. *Effect of GSH, GSSG and cysteine*

	Additions to manometers (ml.)				
Enzyme	0·8	0·8	0·8	0·8	0·8
M/10 $NaHCO_3$	0·4	0·4	0·4	0·4	0·4
Phenylglyoxal	0·4	0·4	0·4	—	0·4
GSH 2 mg./ml.	1·4	—	—	—	—
GSSG 4 mg./ml.	—	1·4	—	—	—
Cysteine-HCl 1 mg./ml.	—	—	1·0	1·0	—
Water	0·2	0·2	0·6	1·0	1·6
CO_2 output, μl./15 min.	202	9·5	127·5	3·0	33

IV. *Substrates.* Glyoxalase attacks several keto-aldehydes. The bacterial enzyme decomposed both methyl- and phenyl-glyoxals. Hayashi [1929] reported that *Bact. coli* attacked phenylglyoxal much less rapidly than methylglyoxal. This did not appear to be the case with the extracted enzyme.

Under suitable conditions the reaction proceeds to completion and the acid can be estimated. In one such experiment 540 μl. CO_2 were evolved, representing 92·5 % of theory. Lactic acid was estimated by the method of Friedemann & Graeser [1933]; 2·08 mg. were found, 2·17 mg. being the amount expected.

SUMMARY

Cell-free extracts of *Bact. coli* contain the enzyme glyoxalase, with properties essentially the same as those of the enzyme obtained from *B. pasteur* and yeast.

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39. ADENYLIC DEAMINASE ACTIVITY IN B_1-AVITAMINOSIS AND IN STARVATION

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(*Received 15 January 1940*)

In our previous studies of carbohydrate metabolism in vitamin B_1 deficiency [Lu, 1939] it was shown that the high pyruvate level of the blood, though running closely parallel with the bradycardia, could not be regarded as directly responsible for it. Other possible causes therefore demanded consideration.

Two series of observations led us to examine the adenylic deaminase activity of pigeon breast muscle during B_1-avitaminosis. In the first place, Birch & Mapson [1936] comparing hearts of normal and B_1-deficient rats, had reported a decrease of about 20·% in the deaminase activity of the deficient hearts; 18 hr. after administration of the vitamin, the hearts of the recovered animals showed normal activity. These authors were in fact interested in the phenomenon from the point of view of the causation of the bradycardia of deficient animals, which resembles the bradycardia caused by injection of adenylic compounds into normal animals. Secondly, Bodnar & Karell [1932], using fluoride-poisoned brei from breast muscle of pigeons deficient in the B complex, had observed greater power than normal of accumulating phosphoric esters. Similar observations were made by Nagai [1932]. Since the presence of adenylic compounds is essential for ester formation, the greater production in the deficient animals might depend upon the higher concentration of adenylic compounds, and might be regarded as an indication suggesting loss of deaminase activity when the vitamin is absent. From the point of view both of the possible connection between adenylic acid and bradycardia, and of the disturbances in deficient muscle metabolism, it seemed of interest to study deamination of adenylic acid and phosphorylation of carbohydrate in skeletal and heart muscles of deficient animals.

Changes in adenylic deaminase activity in pigeon breast muscle

In order to measure the activity of the enzyme, a small portion of breast muscle (0·5–1·0 g.) was weighed, then ground in an ice-cold mortar with 10 vol. of ice-cold *M*/10 phosphate at *p*H 7, a few grains of Merck's quartz being added. After standing 30 min. at 0° for extraction of the enzyme, the extract was strained through muslin. In order to obviate uncertainties due to possible synthesis of added adenylic acid to adenylpyrophosphate (owing to any carbohydrate breakdown in the extract) the extract was always dialysed at 0° against 0·5 % KCl overnight before use; no change in volume took place during this dialysis. The activity of the extracts was tested in the following way: samples containing 1·0 ml. extract, 1·0 ml. phosphate buffer (0·16 *M*, *p*H 6·5), 1·0 ml. adenylic acid (neutralized, and containing 3 mg. adenylic acid) and 2·5 ml. water were mixed, and incubated for 20 and 40 min. at 37°. The NH_3 formation was estimated by the distillation method of Parnas & Heller, followed by nesslerization. The dialysed extracts were diluted before use in the tests, usually 1:10. The NH_3 production observed in the tests was therefore multiplied by 100 to obtain the production per g. muscle.

Preliminary experiments showed that the optimum *p*H lay at 6·5, and this *p*H was therefore always used.

In the earlier experiments, only one test of deaminase activity was performed on each bird, which was killed at the time of the removal of the muscle. These results, while definitely lower on the average in the deficient birds than in the normal, showed considerable variation within the two groups. The method of biopsy was therefore later adopted; a piece of muscle (30–60 mg.) was removed from the breast under ether anaesthesia, and the small wound sewn up. The birds recovered well. Not more than three operations were carried out on the same bird. Control experiments showed that pieces of muscle removed at intervals of some days from a bird kept on normal diet showed no variation in deaminase content; and further that the deaminase activity was the same whether the muscle was removed under ether, or without ether when the animal was killed. The weighed fragment of muscle was in each case extracted with 100 vol. of *M*/10 phosphate; this extract was used in the tests without further dilution.

The deficient diet usually consisted of thoroughly washed polished rice plus one drop of radiostoleum per day. The control birds received the same, together with 15 I.U. of vitamin B_1 each per day. When the deficient birds were to be recovered, they were usually put on to full stock diet (mixed pigeon corn) as well as receiving the amounts of B_1 recorded. The controls were put on to stock diet at the same time. In some cases, the effect of giving B_1 injections to the deficient birds, while continuing the rice diet, was tried. In some later experiments, as is pointed out in the appropriate places, a modified Carter diet was used. This consisted of 15 % Glaxo, 75 % rice starch, 5 % fresh butter and 5 % salts. It was supplemented by 0·5 g. autoclaved marmite, and one drop of radiostoleum every second or third day. This diet is not readily eaten by pigeons; but if they are provided with no other food, and if they receive B_1, they will accept it after a few days. All the birds in an experiment were therefore given B_1, until they were eating the diet well and maintaining weight. Then B_1 was removed from the diet of those destined for deficiency. The degree of this deficiency was judged (*a*) by onset of head retraction; this occurred fairly regularly in the earlier experiments, but much more variably later; (*b*) by heart rate; this symptom also is well known to be very unreliable in pigeons; (*c*) by the great loss of weight and weakness of the birds. With regard to the heart rate, Dr L. J. Harris, to whom we are indebted for much valuable advice, informed us that using the modified Carter diet he has succeeded in getting the heart rate reliably down, and up again with B_1; on this diet he found head retraction (which is probably a symptom of chronic B_1 deficiency) much rarer; the birds may die from heart failure in a shorter time and before head retraction comes on [Harris, 1939].

The curves in Figs. 1 and 2 show the results of the earlier series of experiments. It can be observed that the normals form a group with a considerably higher average than the deficients. The results for the recovered birds show an even wider scatter, and their average is intermediate between that of the other two groups.

In the later work, a series of experiments was done on each bird, muscle being removed at different stages of deficiency or during recovery.

Since B_1-avitaminosis leads to loss of appetite, great reduction in food intake and very serious wasting of the muscle, it was necessary to find out whether low diet plus B_1 led to change in deaminase activity of the muscle. Control birds were therefore used, paired with the deficient birds, so that each control received only as much food as the deficient partner had eaten on the previous day. The controls received 15 I.U. B_1 by injection per day. The importance of paired

feeding is now becoming generally recognized in nutritional investigations [cf. McDonald & McHenry, 1939; Follis *et al.* 1940].

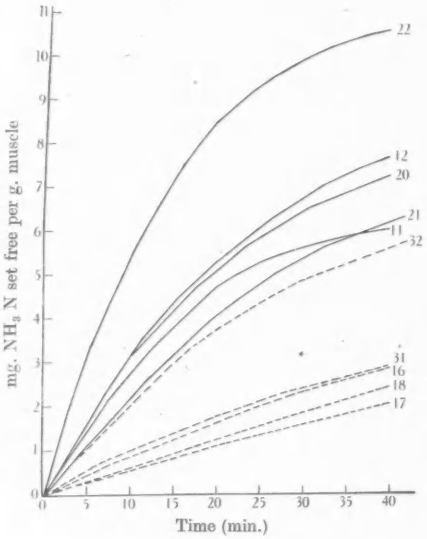

Fig. 1.

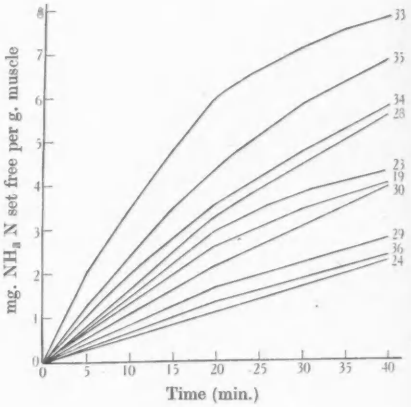

Fig. 2.

Fig. 1. All birds on polished rice diet. —— Normal. ---- Deficient.

Pigeon no.	Notes on deficient birds
16	Marked head retraction.
17	Marked head retraction. Heart rate 140.
18	Head retraction. Heart rate 195.
31	Marked head retraction.
32	Marked head retraction.

Fig. 2. All birds on polished rice diet till markedly deficient. Transferred to stock diet when B_1 was administered.

Pigeon no.	Notes on recovered birds.
28	Heart rate 240. 1 mg. B_1 per day given for 2 days and killed on the 3rd day. Heart rate 480.
29	Heart rate 245. 4 doses of B_1 given. Heart rate rose to 420 on the 2nd day; on the 5th day it was 220. Killed 5th day.
30	Heart rate 175. 1 dose of 0·3 mg. B_1. Killed next day. Heart rate 300.
19	Severe head retraction. 0·3 mg. B_1 per day for 4 days. Killed 5th day. Heart rate 285.
23	Received 2 mg. B_1. Killed next day. Heart rate 240.
24	Heart rate 240. Received 1 mg. B_1 each day for 5 days. Killed 6th day. Heart rate 450.
34	Head retraction. 0·3 mg. B_1 each day for 2 days. Killed 3rd day. Heart rate 280.
35	Received 0·3 mg. B_1. Interval of 1 day. Then 1 mg. a day for 2 days. Killed next day. Heart rate 180.
33	Marked head retraction. 1 mg. B_1 per day for 2 days. Killed 3rd day. Heart rate 310.
36	Heart rate 154. 0·3 mg. B_1. Interval of 2 days. Then 1 mg. per day for 4 days. Killed next day. Heart rate 430.

The results show in every case a marked fall in deaminase activity in the same bird when examined before and after production of avitaminosis (Figs. 3–8 and 10–11). In every case of semi-starvation plus B_1 administration, a marked fall was also found; this fall was not so great as in the deficient birds but neither

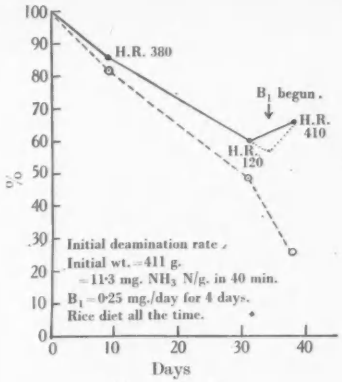

Fig. 3. No. 38.

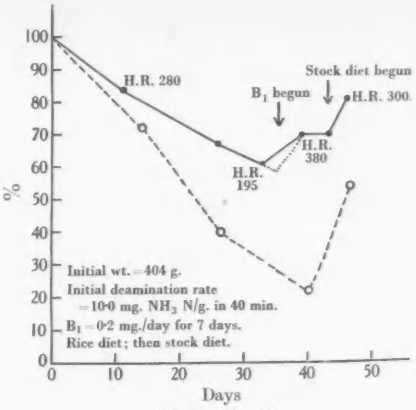

Fig. 4. No. 42.

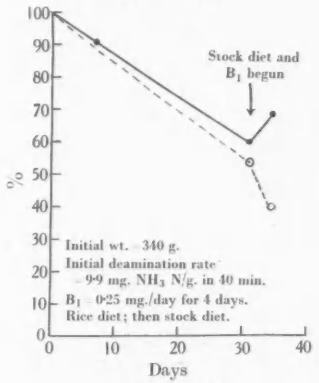
Stock diet and B_1 begun
Initial wt. = 340 g.
Initial deamination rate
= 9·9 mg. NH_3 N/g. in 40 min.
B_1 = 0·25 mg./day for 4 days.
Rice diet; then stock diet.
%
Days

Fig. 5. No. 43.

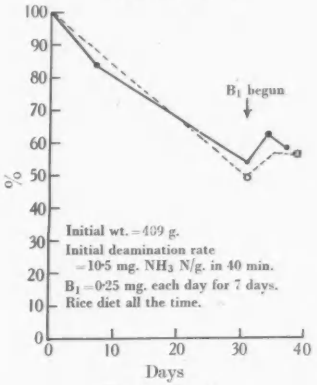
B_1 begun
Initial wt. = 409 g.
Initial deamination rate
= 10·5 mg. NH_3 N/g. in 40 min.
B_1 = 0·25 mg. each day for 7 days.
Rice diet all the time.
%
Days

Fig. 6. No. 44.

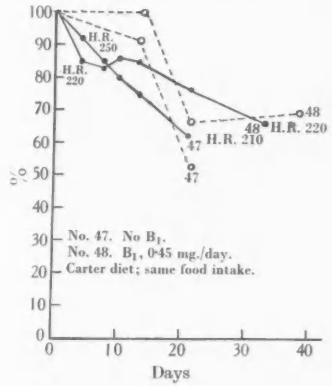

Fig. 7.

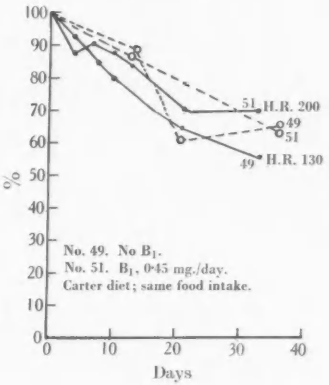

Fig. 8.

In Figs. 3–11 —— indicates body weight; – – – indicates deamination rate; H.R. indicates heart rate.

was the loss of weight so great, in spite of the fact that the food intake was the same (Figs. 7–11).

This suggests that the loss of deaminase activity depends rather on the starvation condition than on the absence of B_1. The results of experiments on recovery substantiate this view (Figs. 3–6, 9, 10). For although return of deaminase activity is sometimes seen in these experiments after administration

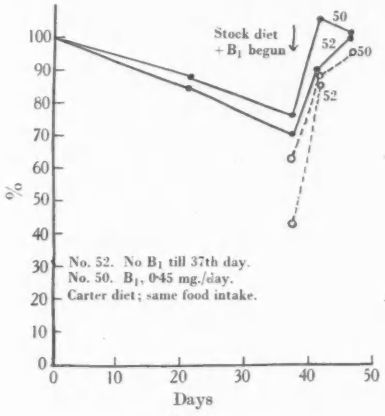

Fig. 9.

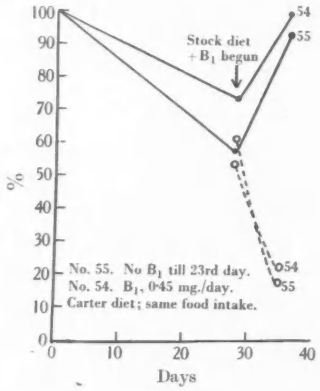

Fig. 10.

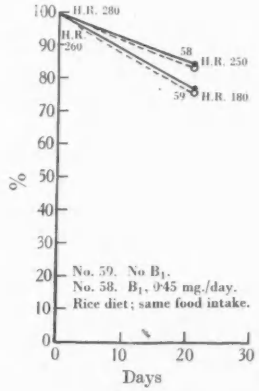

Fig. 11.

of B_1, it occurs to a marked extent only when the animal is rapidly recovering weight. If for example B_1 is given but the rice diet is continued, there is usually a rise in heart rate and general improvement in the condition of the animal; but after an initial rise the weight may remain stationary or fall slightly again. If, on the other hand, the administration of B_1 is accompanied by a change to the stock diet, there is rapid increase in weight, which may return to normal in about 4 days. It is only under the latter conditions that marked recovery of deaminase activity has been observed, and even under these conditions there

were cases where the deaminase activity continued to fall while the muscle weight was being rapidly regained. This question is further discussed later.

In a few cases the B_1 content of the muscle and liver tissue was estimated by the thiochrome method.

	Liver μg. B_1 per g.	Muscle μg. B_1 per g.
No. 49 (killed in deficient state)	0·06	<0·03
51 (semi-starved but receiving B_1)	2·3	1·1
56 (semi-starved but receiving B_1; last dose day before death)	3·4	2·5

These results show that, in spite of the great wastage of tissue going on, the animals receiving B_1 retain it in their organs.

Changes in phosphorylation activity in pigeon breast muscle

The results of Bodnar & Karell [1932] on increased esterification of phosphate in B_1-deficient muscle were confirmed. We used the same conditions as theirs, except that the samples were kept at 37° for 40 min. instead of for some hours at room temperature. 1 g. brei was treated with 0·5 ml. 1 % Na_2HPO_4; 0·5 ml. 2 % NaF and 15 mg. glycogen. The mean of eight experiments on normal birds gave a value of 0·72 mg. P esterified in 40 min. (limits 0·4–1·0); while the mean for nine deficient birds was 0·98 mg. (limits 0·7–1·3). These differences are not so large or so constant as those found by Bodnar & Karell—their averages were 0·70 mg. for normal and 1·25 mg. for deficient animals.

In the brei inorganic P is disappearing, both by the Parnas esterification of glycogen with formation of hexosemonophosphate, and the 'coupled' esterification via adenylic acid with formation of hexosediphosphate. Some experiments were done to see whether the phosphorylase system showed different activities in the two cases. Muscle extract was made by crushing the muscle with an equal volume of ice-cold water; in order to obtain esterification by the phosphorylase system alone two methods were adopted:

(1) The extract was allowed to stand 24 hr. before use.

(2) The fresh extract was incubated with $M/250$ iodoacetic acid for 15 min. before addition of substrate, to bring about inhibition of the 'coupled' mechanism.

In both cases, to 1 ml. of extract were added 0·5 ml. 0·33 M phosphate at pH 7·0 and 15 mg. glycogen.

The average result for five extracts from normal birds by the first method was 0·58 mg. P esterified per ml. in 45 min.; for six deficient birds the average was 1·05 mg. By the second method the average for four normal birds was 0·66 mg., for five deficient birds 0·89 mg.

It thus appears that phosphorylase activity is enhanced to some extent in the B_1-deficient animals. In this connexion it is interesting that although muscular weakness and dystrophy exist also in vitamin E deficiency, the phosphate esterification is there distinctly decreased [Lu *et al.* 1940].

Adenylic deaminase and vitamin B_1 in vitro

Before it became likely that the connexion between the enzyme and the vitamin was an indirect one, we made some tests to find whether addition of the vitamin or cocarboxylase to the deficient extract would restore the deaminase activity to normal. But although we tried many experimental variations, we were never able to observe any such activating effect.

Changes in deaminase activity in rat leg muscle

Similar experiments to those on the pigeon breast muscle were carried out on rat leg muscle. In each case all the muscles of one leg were removed, chopped and well mixed, before a portion was weighed out. The results are given in Table 1.

Table 1. *Change in deaminase activity of normal and deficient rat leg muscle*

	No.	Heart rate	Deamination mg. NH_3 N set free by 1 g. muscle in 40 min. at 37°
Normal	II	—	2·24
	XIII	534	2·85
	XIV	500	2·25
	XV	540	3·60
	XVI	520	3·70
	XVII	540	3·35
	XVIII	570	3·63
	XXVII	490	2·17
	XXVIII	520	2·37
			Average 2·90
Deficient	III	—	3·36
	IV	—	2·35
	V	375	2·34
	VI	300	2·09
	VII	385	2·27
	VIII	320	2·03
	XIX	—	2·12
	XX	—	2·40
			Average 2·37
Recovered	IX	475	2·85
	X	—	1·95
	XI	490	1·89
	XII	530	3·03
			Average 2·43

It is obvious that the decrease in deaminase activity is not nearly so marked as in pigeon muscle; on the average it does not exceed 20 %. It is to be observed that even this small difference between the normal and the deficient depends on the high values in rats nos. XV–XVIII, all of which belonged to one group.

Adenylic deaminase activity in rat heart muscle

In a number of the rat experiments just described, the deaminase activity of the hearts was also tested, and to our surprise, no significant difference between the three groups could be detected. A series of experiments was therefore carried out, using similar conditions to those of Birch & Mapson [1936], to whom we are indebted for information about their experimental details. The heart was weighed and ground with quartz and 10 vol. of ice-cold water in a cooled mortar. After extracting at 0° for 30 min., the extract was strained off through muslin and allowed to stand at room temperature for 2 hr. in order that any glycolysis in the extract might be completed. 1 ml. of the extract was then mixed with 1·2 ml. *M*/6 phosphate buffer *p*H 7·0, 0·2 ml. neutralized solution containing 6 mg. adenylic acid, and 3·6 ml. water. The samples were incubated at 37° for 20 and 45 min. (Table 2). The difference is only 7 %.

Table 2. *Changes in deaminase activity of normal and deficient rat heart muscle*

	No.	Heart rate	Deamination mg. NH_3 N set free by 1 g. heart in 45 min. at 37°
Normal	33	550	0·68
	34	515	0·62
	42	500	0·73
	43	520	0·71
	46	480	0·64
	47	500	0·62
	54	510	0·52
	53	480	0·60
			Average 0·64
Deficient	35	410	0·64
	36	390	—
	37	380	0·77
	38	390	0·65
	39	314	0·65
	40	130	0·74
	41	220	0·73
	44	320	0·66
	45	350	0·70
	55	280	0·68
	52	300	0·76
			Average 0·69

The effect of ions on the adenylic deaminase

While this work was in progress a paper by Conway & Cooke [1939] appeared in which the powerful effect of certain anions, notably phosphate and carbonate, in inhibiting the activity of the deaminase at low concentrations of substrate was described. It seemed necessary, therefore, especially as our substrate concentrations were low (0·06 %), to include examination in citrate buffer in determining the activity of the enzyme. Extra samples were set up in which the *M*/30 phosphate buffer was replaced by *M*/20 citrate. With pigeon muscle it was found that deamination is about 3·5 times as fast in citrate buffer under our conditions; with rat muscle, about 8·5 times as fast. The effect of the citrate buffer appeared to the same extent with both normal and deficient muscle extracts. Conway & Cooke suggest that the effect of the phosphate ion may be explained by assuming adsorption of the anion on the enzyme with exclusion of the adenylic acid, which would agree with the diminished inhibitory effect found by them with increasing substrate concentration in rabbit muscle extract. Similar results were obtained by us on rat muscle extract. In citrate buffer, using 1 : 1250 dilution of muscle extract, at 0·06 % adenylic acid concentration, 9·28 mg./g. NH_3-N were set free in 10 min.; with 10 times the substrate concentration, 0·6 %, the NH_3 formation was approximately doubled—21·0 mg./g. In phosphate buffer, on the other hand, increasing the substrate concentration ten-fold led to a nearly twenty-fold increase in deamination. At 0·06 % adenylic acid, the NH_3-N set free was 1·1 mg./g. in 10 min.; at 0·6 % substrate, 18·65 mg./g.

It may be noticed that in citrate buffer, where presumably the enzyme is maximally active, there is little difference between the deamination rates of pigeon and rat muscles; in the rat muscle extract the enzyme shows a greater degree of inhibition by phosphate.

The rates of deamination in rat heart muscle were also compared in phosphate and citrate buffers. Very little difference was found.

	NH_3-N mg./g.	
	In phosphate	In citrate
Normal heart	0·68	0·74
Deficient heart	0·62	0·58

The bulk of the heart consists, of course, of ventricle. According to Conway & Cooke [1939] ventricular tissue in the rabbit contains an adenosine deaminase but little if any true adenylic deaminase, although the latter is present in the auricular tissue. The adenosine deaminase was found by them not to be inhibited by phosphate. The fact therefore that the rat heart shows little difference of activity in different buffers suggests that here too an adenosine deaminase may be concerned. Conway & Cooke propose two methods whereby it may be tested whether adenylic acid is directly deaminated, or only after initial dephosphorylation.

(1) Adenylic deaminase of animal tissues seems to be specific for muscle adenylic acid, but if the deamination is indirect, by means of adenosine deaminase, the rates of deamination of yeast and muscle adenylic acids will be the same (assuming the rates of dephosphorylation to be the same). This method was tried on rat heart, but it was found that yeast adenylic acid was practically untouched. This result is not conclusive for the presence of true adenylic deaminase, as it may only mean that the rates of dephosphorylation differ in this case.

(2) The NH_3 set free after addition of adenosine to saturate the adenosine deaminase may be compared with the amounts set free after addition of adenosine and adenylic acid together.

The following experiment was performed. The extract was made as usual from normal hearts. The adenosine solution contained 12·5 mg./ml.; the adenylic acid solution 30 mg./ml.; the buffer was *M*/6 phosphate, *p*H 7·0.

	1	2	3	4	5
Extract	1·0	1·0	1·0	1·0	1·0
Buffer	0·6	0·6	0·6	0·6	0·6
Adenylic acid	0·2	0	0	0·2	0·2
Adenosine	0	0·4	0·8	0·4	0·8
NH_3 formation, mg. N/g. tissue in 20 min.	0·029	0·057	0·054	0·054	0·054

These results show that adenosine deaminase is present and saturated with substrate under the conditions used, for doubling the concentration of adenosine leads to no increase in NH_3 formation. That a separate enzyme for deamination of adenylic acid is absent, is shown by the fact that addition of adenylic acid to the system has no effect on the liberation of NH_3.

Discussion

In this work it has been established that during the inanition accompanying vitamin B_1 deficiency in the pigeon, skeletal muscle suffers a loss in adenylic deaminase which may be as great as 75 %. In two or three cases it was even 90%. That the changes cannot be due to changes in water content of the muscle is shown by the results of Roche [1924], who found the water content of normal pigeon breast muscle to be 74·8 %, of avitaminotic birds (on polished rice diet) 73·6 % and of birds in inanition 75·3 %.

The facts that injection of vitamin B_1 leads to no rapid increase in deaminase activity, that the fall occurs on a low diet even if vitamin B_1 is injected and

that vitamin B_1 has no activating effect on the enzyme of the deficient extract *in vitro*, all indicate that the vitamin is not playing here any sort of coenzyme role, as its pyrophosphate does in the oxidation of pyruvic acid.

It may be interesting to compare the effect of vitamin B_1 deficiency on deamination with its effect on respiration. Roche [1924] found that respiration of the isolated muscle tissue was the same in normal, deficient and starved birds, and he did not observe any fluctuations due to rice in the crop, etc. Galvao & Pereira [1937] found that breast muscle tissue from vitamin B_1-deficient pigeons may have a very low respiration, even after administration of B_1 to the bird. On the other hand, the respiration of the muscle from the recovered animals might rise to normal, and this return seemed to be correlated with the ingestion of food, or with the fact that the birds, in becoming deficient, had not lost excessively in weight. The suggestion is that the intensity of tissue respiration observed depends not only on the presence of the vitamin, but also on availability of substrate.

The same conclusion is reached by Lipschitz *et al.* [1938] in their work on the pyruvate oxidation by brain, kidney and liver, in vitamin B_1-deficient chicks. They found that the ability of kidney, liver and brain tissue from polyneuritic birds to oxidise added pyruvate was impaired. The feeding of glucose to the deficient birds led to a rise in the power of the liver to remove pyruvate, though normal values were not reached. The liver and kidney tissue from fasted birds also showed a decreased power to remove pyruvate, and the oral administration of glucose to the fasted birds led to restoration of the ability of the liver tissue to remove pyruvate. Thus it seemed that both with polyneuritic and fasted birds, the provision of some other substrates (derived from glucose) was involved in the pyruvate removal. In the fasted birds, the provision of these substrates seemed to be the only factor necessary; with the polyneuritic birds, complete restoration of the power to remove pyruvate was not attained with glucose only.

In the case of deamination it seems that the explanation of the loss of deaminase activity is not loss of coenzyme (see above) or lack of metabolites, for in all cases the activity is tested in greatly diluted dialysed extracts; the suggestion arises that the enzyme is actually lost from the tissue. In the great loss of material that goes on from the muscle, all the protein fractions may not be involved in exact proportion to their concentration in the muscle; it would not be surprising if, in the substance broken down and removed, some enzymes were involved more than others. That some enzymes escape destruction to a disproportionate degree is suggested by Nagai's finding [1932] that glycerophosphatase activity is greater by about 20 %, and hexosediphosphatase activity by about 40 % in the deficient muscle. But phosphate metabolism in a muscle brei is so complex that a number of factors may be influencing the accumulation of phosphate in these cases just as they may be influencing esterification of phosphate in the phosphorylation experiments mentioned earlier.

If the protein of the deaminase enzyme has to be resynthesised after recovery, the conditions governing its reappearance in full activity may be very complex. The muscle might increase greatly in weight and in protein content, without proportionate formation of the enzyme, and this state of affairs might account for the cases (such as 54 and 55, Fig. 10), where, after administration of the vitamin and stock diet, there is rapid increase in weight accompanied by still further fall in deaminase activity.

The function of the adenylic and adenosine deaminases in general, and their relation to the regulation of heart beat in particular, are obscure. Their function, especially that of the very active adenylic deaminase in skeletal muscle, has

often been regarded as one of detoxication, and Birch & Mapson [1936] regarded even a 20 % decrease in the activity of the heart enzyme as a possible explanation of the bradycardia of B_1 deficiency. As we have found, however, this fall in heart deaminase activity does not always accompany B_1-avitaminosis, even when the bradycardia is as well marked as usual; and, moreover, the heart rate may return to normal while the deaminase of the skeletal muscles does not do so (see e.g. Figs. 3 and 4).

Conway & Cooke [1939] have pointed out that in spite of the deaminases of the tissues, an adenine nucleotide (other than muscle adenylic acid) exists in blood plasma, to the extent of about 5–10 mg./100 ml.; red blood cells contain adenylpyrophosphate and adenosine, and are freely permeable to the adenylic acid which is probably formed there. There is no evidence as to the concentrations of adenylic acid compounds normally reaching the sensitive sites of the heart, or what increase in concentration is necessary at these sites for the effects of bradycardia to be produced.

Conway & Cooke have remarked that the effective deamination rate in skeletal muscle is 500–1000 times that in any other tissue. NH_3 is also formed in skeletal muscle from adenylic acid 20 times as fast as it is from adenosine by the tissues with the most active adenosinase (appendix, jejunum). It seems that considerable quantities of adenine nucleotides must escape from the intestine into the blood; and if this is the case it becomes difficult to regard the skeletal deaminase as a mechanism to protect the rest of the body from effects of adenylic acid. If the adenylic deaminase has some function within the muscle itself, this is at present unknown.

Summary

1. Muscle tissue of animals in vitamin B_1 deficiency shows a diminution of the activity of adenylic deaminase. This is especially marked in pigeon breast muscle, less so in rat leg muscle. Differences between these animals in the absolute levels of their deaminase activities may be largely due to ionic inhibition effects as discussed in the text. Rat heart muscle deaminates adenylic acid indirectly. The latter is first dephosphorylated, and then NH_3 formation is caused by action of adenosine deaminase. No difference was observed in rate of NH_3 liberation between normal and deficient heart muscles.

2. Decrease in the adenylic deaminase activity is accompanied by some increase in phosphorylase activity in the deficient muscle.

3. Paired feeding experiments with inanition unaccompanied by vitamin B_1 deficiency show, however, that the diminution of deaminase activity is primarily connected with the starvation, and only secondarily with vitamin deficiency. The return to normal is slow, requires normal food intake and cannot be brought about by vitamin B_1 alone.

4. Bradycardia, on the other hand, gives place rapidly to normal heart rate as a result of administration of B_1 alone. It seems unlikely, therefore, that the bradycardia is due to accumulation of adenylic acid following the decreased deaminase activity.

The thanks of the authors are due to Dr Leslie J. Harris for much valuable advice and assistance; to Mr Ying-Lai Wang for carrying out thiochrome estimations; to Dr H. Lehmann for advice on biopsy technique; and to Miss R. Leader, Mr H. Mowl and Miss L. Gordon for technical assistance.

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40. THE CYSTINE AND METHIONINE IN CERTAIN SPECIES OF GRASSLAND HERBAGE

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(*Received 3 March 1941*)

It would appear from recent investigations that both of the amino-acids containing sulphur are to be regarded as essential in animal nutrition, so that it is important to have data on the amounts of these acids in animal foodstuffs. Grass is the most important source of protein to the animal, and the object of the present investigation was to determine the cystine and methionine in a few of the more important species of grassland herbage. Most of the work with the grasses has been carried out by Chibnall and his co-workers, who have made fairly comprehensive analyses of the protein preparations of the leaves of many plants.

Experimental

Material examined. Attention was confined to four species, viz. white clover, *Trifolium repens*; cocksfoot (Danish), *Dactylis glomerata*; perennial rye-grass (Ayrshire), *Lolium perenne*; and timothy (Scotch), *Phleum pratense*. Samples were taken at different stages of growth from different places during two seasons. Most of the farms were in late districts and in rather exposed areas, so that there was not much growth of grass before May when the first samples were collected. One farm was situated on deep peat which had recently been reclaimed by drainage and had received liberal dressings of lime and fertilizers. The other farms were on mineral soils varying in texture from light to heavy loams and in *p*H value from about 5 to 7·5. The readily available potassium and phosphorus varied from very low to moderately good values. A few experiments with fertilizers were incorporated in the plan of the work, but, as is shown below, the results were relatively unimportant. In some cases the samples were obtained from plots of pure species, but usually the herbage was mixed and the individual species were separated by hand. The plants were cut about 1 in. above ground level with a knife and the fresh material was transported to the laboratory in waterproof bags and examined immediately.

Separation of protein. The preparation of a reasonably pure protein from the leaves of plants has engaged the attention of many workers and the question is fully dealt with by Chibnall [1939]. In the present investigation, it was decided to employ the method of extraction using a slightly alkaline buffer solution as suggested by Lugg [1939]. Between 300 and 400 g. of the fresh plant material were disintegrated twice in a mincing machine with about three times that quantity of a solution of borax containing 1·1 g. boron per litre and of *p*H 9·2. The cell-wall debris was removed by squeezing the pulp in a linen bag and the colloidal extract was filtered through paper on a Büchner funnel. The

*p*H of the filtrate was brought to about 4·5 by the addition of 4 ml. 2*N* H_2SO_4 per 100 ml. when the protein settled out quite quickly. Next day the supernatant liquid was siphoned off and the precipitate was washed twice with water. The protein was then separated by centrifuge, washed four times with absolute alcohol and twice with methylated ether and then extracted for 20 hr. in a Soxhlet apparatus with light petroleum (B.P. 40–60°). This final extraction was found to be necessary to remove traces of ether-soluble material which interfered with the subsequent determination of methionine. The protein preparation was finally dried over P_2O_5 in a vacuum.

Protein preparations

Yield and total nitrogen. The percentage of N in the protein preparations was rather irregular. It varied from 10 to nearly 14% for white clover, from about 9 to 14% for cocksfoot, from 6 to 13% for rye-grass and from 8 to about 13% for timothy. The success of the extraction naturally depends upon the thoroughness of disintegration and probably upon the nature of the protoplasm. The clover yielded the most protein, the recovery of the N in the original sample varying from 24 to 43%. Cocksfoot on the other hand yielded the least protein, the preparation accounting for only 15 to 34% of the total N. Within each species, more protein was usually obtained from the young and aftermath samples than from the samples of older material. The results are summarized in Table 1, in which the samples have been grouped according to stage of growth rather than to date of sampling.

Table 1. *N and amino-N (after hydrolysis) in fat-free protein preparations from white clover (W.C.), cocksfoot (C.), perennial rye-grass (R.) and timothy (T.)*

	% of N in protein preparation				N in protein preparation as % of total leaf-N				% total N in protein preparation as amino-N after hydrolysis			
Nature of sample	W.C.	C.	R.	T.	W.C.	C.	R.	T.	W.C.	C.	R.	T.
Young	10·5	12·7	10·0	8·0	38	29	21	*10	66	61	60	58
	13·8	—	—	—	*6	—	—	—	65	—	—	—
	12·8	—	—	—	37	—	—	—	68	—	—	—
Flowering or heading	10·2	10·6	10·4	10·7	*16	24	24	31	71	60	61	64
	10·0	11·0	12·0	10·8	31	26	22*	25	59	57	66	62
	10·9	12·9	9·6	—	28	22	25	—	63	62	68	—
	—	14·1	12·8	—	—	26	24	—	—	63	60	—
Old	†10·2	9·7	6·0	11·1	30	20	25	*20	73	72	53	67
	11·7	9·9	8·6	—	31	17	22	—	68	63	63	—
	13·2	11·6	—	—	24	21	—	—	72	63	—	—
Aftermath	11·1	11·0	†8·1	†12·5	34	26	30	29	66	64	57	57
	11·9	11·2	10·2	8·5	27	21	32	33	68	64	59	57
	—	10·1	11·0	—	—	23	34	—	—	61	59	—
	—	†8·6	10·3	—	—	20	28	—	—	70	57	—
	—	10·9	10·7	—	—	27	27	—	—	62	62	—
	—	10·4	—	—	—	15	—	—	—	64	—	—
Grazed	13·8	9·5	10·8	8·6	43	34	26	31	61	63	58	62
	13·0	11·6	12·5	11·8	32	32	29	34	70	63	63	62
	—	—	13·1	—	—	—	24	—	—	—	58	—
	—	—	12·3	—	—	—	25	—	—	—	62	—
Average	11·8	11·0	10·5	10·3	—	—	—	—	67	63	60	61
Ash content	4·8	6·8	7·6	4·3	—	—	—	—	—	—	—	—

* A portion of protein lost during manipulation.
† Samples subjected to determination of ash.

The figures for the N recovered as protein are affected, of course, by the inevitable losses of material during extraction and consequently give only a general idea of the extent of the extraction. In the case of two samples of rye-grass, the residue from extraction was re-extracted and the protein precipitated and prepared in the usual manner. The amount obtained was very small, being only about a fifth of the first preparation, but there was enough to make determinations of total N and amino-N after hydrolysis. A comparison of these figures for the first and second preparations indicates that there is no striking difference between the protein obtained in the first extraction and that left in the residue and lends support to certain observations made by Chibnall [1939].

	Total N in protein preparation		Amino-N after hydrolysis as % of total N	
Sample	1st extract	2nd extract	1st extract	2nd extract
1	11·7	10·4	63	63
2	11·9	9·8	57	57

Ash. The total ash was determined in only one sample of each species and the results varied from 4·3 to 7·6%.

Amino-N. The total amino-N was determined by the Van Slyke [1915] gasometric method using a micro-apparatus. Some preliminary tests were carried out with glycine to find the time of shaking required to give a maximum yield of amino-N. Glycine gives high but consistent results and a maximum recovery of 111·8% was obtained with 8 min. shaking at about 300 r.p.m.; this figure is similar to that obtained by Levene & Van Slyke [1912], and a period of 8 min. shaking was adopted for further work. Five other amino-acids were examined; a small quantity of each was dissolved in water (a little dil. HCl was used with cystine and tyrosine) and made up to a definite volume from which 2 ml. were taken for each test. The average results are given in Table 2 and compared with those obtained by Van Slyke [1911] for the same compounds.

Table 2. *Average percentage recovery of amino-N*

	Arginine	Cystine	Histidine	Lysine	Tyrosine
Present investigation	102·3	108·5	103·0	86·9	102·4
Van Slyke [1911]	99·8	107·4	98·2	95·4	102·1

It will be observed that the results for arginine, histidine and tyrosine are not far removed from the theoretical values, whilst that for cystine is much higher and that for lysine rather low. The amounts of glycine, cystine and lysine in grass proteins are relatively small, however [Chibnall, 1939], so that the error in the total amino-N estimated by the above procedure is unlikely to be serious.

The grass proteins were hydrolysed with acid; a few preliminary experiments showed that a maximum percentage of amino-N was obtained when about 1 g. of the preparation was boiled with 25 ml. of 3*N* HCl for 8 hr. The yield of amino-N was not affected by the previous addition of known amounts of amino-acid to the protein preparation. Figures for the proportion of amino-N in the different preparations are included in Table 1. There are no important differences between the protein preparations of the four species with regard to the percentages of total amino-N; the figures for clover vary from 59 to 73, for cocksfoot from 57 to 72, for rye-grass from 53 to 68, and for timothy from 57 to 67. It will be observed, however, that the amount of amino-N tends to increase as the plant becomes ripe—a fact which is particularly noticeable when a comparison is made between samples taken from the same farm. Generally speaking, it may

be said that the amount of amino-N in the hydrolysed samples of all four species reached a maximum during July.

Manurial treatment. The effects of manurial treatment were rather irregular. Lime increased the amino-N in both cocksfoot and rye-grass, but with slag the results obtained for cocksfoot were the reverse of those obtained for rye-grass. A late application of ammonium sulphate, three weeks before cutting, produced a considerable decrease (about 12%) in the amino-N of rye-grass protein, although the total N in the grass was increased by about 40%.

Sulphur-containing amino-acids

The method used in the present investigation was based on the procedure devised by Baernstein [1936] and involved hydrolysis with HI.

Apparatus. A few modifications were made of the apparatus used by Baernstein. The digestion flask was connected to the condenser by a ground joint which seemed to be tight enough without the mercury seal suggested by Kassell & Brand [1938]. The absorbers were connected in series by rubber stoppers and tubing. The first absorber contained 10 ml. of a 1% suspension of red phosphorus in distilled water; the outlet of this tube was packed with glass wool to prevent the phosphorus from being carried over. The second absorber contained 10 ml. of a solution containing 20% $CdCl_2$ and 20% $BaCl_2$ to absorb H_2S and SO_2; the third contained 10 ml. of saturated $HgCl_2$ to remove phosphine from the KH_2PO_2 added to the HI; the fourth contained 10 ml. of 10% potassium acetate in glacial acetic acid with six drops of bromine. This solution absorbs the methyl iodide produced from the methionine and the fifth absorber contained 5 ml. of the same solution to ensure complete absorption of the volatile iodide. The HI was purified by double distillation; its specific gravity was 1·7 and 1% KH_2PO_2 was added to prevent formation of free iodine.

Hydrolysis. About 0·5 g. of the protein preparation was placed in the digestion flask (capacity about 50 ml.), which was then filled with nitrogen gas previously passed through a solution of 5% $AgNO_3$ and concentrated H_2SO_4. A few small pieces of unglazed porcelain, 10 ml. of HI and about 0·02 g. of KH_2PO_2 were then added and the flask was connected to the condenser, in which the water was maintained at about 50°. The absorption train was then assembled and attached to the condenser. The digestion flask was immersed in an oil bath at 150° and the first three absorbers in water at 50–60°. During hydrolysis, a stream of nitrogen was passed through the apparatus at a constant rate controlled by a pinch cock on the side tube of the digestion flask. Maximum values were obtained when the boiling and aeration were continued for 5–6 hr. for pure protein and for 7–8 hr. for grass protein.

Cystine. At the end of the digestion, the absorption train was disconnected from the condenser, 0·02 g. of KH_2PO_2 was added to the digestion flask and, with suction from a pump at the condenser outlet, the digest was concentrated to about 3 ml., the stream of nitrogen being continued meantime. After the digest had cooled in a current of nitrogen it was made up to 25 ml. with 4% HCl. 10 ml. were pipetted into a conical flask filled with nitrogen and a few ml. of $N/100$ $KIO_3.HIO_3$ were added until the solution was definitely orange in colour. The excess iodine was titrated with $N/100$ $Na_2S_2O_3$, using starch as an indicator.

Methionine. The methionine, which has been converted during the hydrolysis into the lactone of homocysteine, was now determined in the same solution. A few drops of bromothymol blue were added together with one drop of octyl alcohol and a quantity of freshly prepared 0·04N $Na_2S_4O_6$ so as to give, with the tetrathionate produced in the cystine titration, an excess of 2·5 ml. 1 ml. of

0·04 *N* $Na_2S_4O_6$ is equivalent to 6 mg. of methionine and the amount of methionine present was already estimated in the volatile iodide determination described below. The flask was fitted with a rubber stopper, carrying two tubes, and filled with nitrogen gas. It was then evacuated and about 2 ml. of concentrated ammonia were drawn in from a burette, inserted through one of the tubes, to make the solution definitely alkaline. The flask was again evacuated and immersed in warm water at 40° for 15 min. It was again filled with nitrogen and the connecting tubes were removed. The digest was acidified with 10 ml. of 5 *N* HCl and the thiosulphate produced during the process of oxidation was titrated with *N*/100 $KIO_3.HIO_3$. A most satisfactory end-point was obtained by adding a slight excess of the biiodate and titrating back with thiosulphate. The methionine was calculated from the iodine required, 1 ml. of *N*/100 $KIO_3.HIO_3$ being equivalent to 0·1407 mg. of methionine-N or 1·49 mg. of methionine.

The methionine was also determined from the volatile iodide absorbed in the fourth and fifth absorbers. The contents of these were rinsed into a 100 ml. volumetric flask containing 25 ml. of 25 % sodium acetate solution. A little formic acid (about 10–12 drops) was added to reduce the bromine, the flask was shaken and the contents made up to the mark. Aliquot portions of 20 or 25 ml. were pipetted out, a little KI and a few drops of 10 % HCl were added and the liberated iodine was titrated with *N*/100 $Na_2S_2O_3$.

Sulphate. The H_2S and SO_2, produced by the reduction of sulphate, have been absorbed in the second absorber as CdS and $BaSO_3$, which may be quantitatively oxidized. The contents of the tube were rinsed into a beaker, a small excess of *N*/100 $KIO_3.HIO_3$ was added followed by a few crystals of KI and 2 ml. of 10 % HCl. After standing for a short time, the excess iodine was titrated with *N*/100 $Na_2S_2O_3$, 1 ml. of the iodate being equivalent to 0·16 mg. of sulphur.

Tests of the method. It was found advisable to keep both the condenser and the first three absorbers warm in order to ensure that the methyl iodide passed to the fourth and fifth absorbers. Lugg [1938, 1] recommended that the digestion of protein be commenced with the addition of 0·02 g. of KH_2PO_2 and that further quantities be added at intervals as long as iodine coloration was obvious. In hydrolysing impure leaf protein, however, the digest is dark orange in colour for reasons other than the presence of iodine, and it was found that the addition of 0·02 g. of KH_2PO_2 at the beginning and end of the hydrolysis was sufficient. Blank determinations were made with the HI at frequent intervals and corresponded to about 0·02–0·04 mg. of cystine-N, 0·03–0·04 mg. of S, 0·01 mg. of methionine-N by volatile iodide and 0·06–0·1 mg. of methionine-N by homocysteine.

In the examination of the method with a sample of edestin, containing 15·58 % N, with and without the phosphorus absorber, and with both cold and warm conditions for the condenser and the first three absorbers, there was not much difference between the figures either for cystine or for methionine by homocysteine, but the percentage of methionine by volatile iodide depended upon the conditions employed. The volatile iodide appeared to be detained when the conditions were cold and it seemed to be advisable to introduce the absorber containing red phosphorus. The percentage distribution of the sulphur in the edestin was found to correspond to 1·09 % cystine-N and 1·24 % methionine-N as compared with figures, obtained by other workers, of 0·89–1·31 for cystine-N and of 1·08–1·35 for methionine-N.

A number of preliminary trials were made of the recovery of cystine and methionine alone or mixed or added to edestin, the analyses being carried out

under the conditions already described. The recovery of cystine averaged 97·5 % when examined alone, 99·3 % in presence of methionine and 98·8 % in presence of methionine and edestin. The recovery of methionine examined alone averaged 95·5 % by the volatile iodide method and 91·1 % by the homocysteine method, and the recovery was not affected by the presence of cystine or cystine and edestin.

With regard to the recovery of pure substances, Baernstein [1936] found values of 101·5 % for cystine and 99·4 and 99·6 % for methionine by the volatile iodide and homocysteine methods respectively. In the present investigation the recovery of cystine was satisfactory but the recovery of methionine was low. This, according to Kassell & Brand [1938], is probably due to the formation of methyl mercaptan. It is noteworthy that a small quantity of cystine, amounting on an average to 5·2 % of the methionine-N, was found in the estimation of methionine, suggesting that a small proportion of the homocysteine was titrated as cystine. When the analysis of a mixture of cystine and methionine was carried out, the recovery of cystine was therefore higher than when cystine was examined alone, while the recovery of methionine was not affected by the presence of cystine. The percentage recoveries were in good agreement with those found by Kassell & Brand [1938], who obtained 97·8 % for cystine and 93·7 and 89·3 % for methionine by the volatile iodide and homocysteine methods respectively.

Since the grass proteins contain both cystine and methionine, the correction factors used in the subsequent analysis were 1·007 for cystine and 1·047 or 1·098 for methionine according to the method of estimation. In the examination of the grass proteins, however, the yield of methionine was always about 25 % higher by the methyl iodide method than by the homocysteine method even with the above correction factors. It must be assumed that the protein preparations were not entirely free from impurities which could yield volatile iodide or could render methionine-sulphur insoluble.

Results

General. The results are all reported (Table 3) on a nitrogen basis, but do not include the figures for ten samples collected from manurial experiments. The distribution of S (Table 4) was calculated on the assumption that cystine and methionine were the only two S-containing amino-acids in the proteins and that the remainder of the S was in the form of sulphate. The figures for the sulphate-S were not corrected for the small amounts of H_2S liberated from cystine and methionine and corresponding to 1·25 and 0·89 % respectively of the cystine-S and methionine-S. The figures for methionine by the homocysteine method are given in Table 4.

The four species do not reveal noteworthy differences in their contents of cystine and methionine. The former varies from 0·90 to 1·40 % for white clover, 0·91 to 1·46 % for cocksfoot, 0·96 to 1·26 % for rye-grass and 0·82 to 1·32 % for timothy. The corresponding figures for methionine-N by homocysteine are 0·70 to 1·20 %, 0·73 to 1·24 %, 0·91 to 1·10 % and 0·74 to 1·29 %. In an examination of ten samples of leaf proteins by the same method, Lugg [1938, 1] found that the cystine varied from 1·1 to 1·5 % and the methionine from 1·2 to 1·6 % and that rather higher values were obtained by his differential oxidation method. Our figures are, therefore, somewhat lower than those obtained by Lugg. It will be observed that the legume, white clover, contained about the same amounts of the amino-acids as the grasses.

Table 3. *Cystine and methionine in grass protein preparations*

Nature of sample	% of total N in protein preparation as cystine- and/or cysteine-N				% of total N in protein preparation as methionine-N							
					By volatile iodide				By homocysteine			
	W.C.	C.	R.	T.	W.C.	C.	R.	T.	W.C.	C.	R.	T.
Young	1·18	1·28	1·04	0·86	1·22	1·59	1·27	1·04	1·04	1·17	1·04	0·78
	1·14	—	—	—	1·26	—	—	—	1·02	—	—	—
	1·03	—	—	—	1·30	—	—	—	0·94	—	—	—
Flowering or heading	1·33	1·37	1·16	1·13	1·40	1·52	1·30	1·17	1·15	1·12	1·10	0·91
	1·32	0·91	1·16	1·32	1·50	1·29	1·50	1·59	1·20	0·98	1·06	1·29
	1·40	1·32	1·26	—	1·46	1·52	1·43	—	1·14	1·20	1·10	—
	—	1·25	1·16	—	—	1·30	1·43	—	—	0·84	0·91	—
Old	1·35	1·46	1·12	1·24	1·38	1·30	1·52	1·53	1·13	1·22	1·01	1·19
	1·20	1·42	1·21	—	1·42	1·46	1·55	—	1·11	1·24	1·10	—
	1·36	1·42	—	—	1·48	1·54	—	—	1·20	1·21	—	—
Aftermath	1·01	1·01	0·96	1·20	1·20	1·21	1·40	1·38	0·90	0·73	1·05	1·12
	1·10	1·25	1·03	0·82	1·23	1·40	1·28	1·34	0·98	1·08	0·97	0·74
	—	1·05	0·98	—	—	1·41	1·34	—	—	0·84	0·89	—
	—	1·34	1·08	—	—	1·28	1·14	—	—	1·07	0·95	—
	—	1·16	1·03	—	—	1·50	1·20	—	—	1·09	0·94	—
	—	1·44	—	—	—	1·46	—	—	—	1·15	—	—
Grazed	0·90	1·13	1·10	0·85	1·29	1·35	1·18	1·40	0·70	1·03	1·00	0·97
	1·04	1·12	1·15	1·25	1·32	1·30	1·25	1·26	1·00	1·03	1·05	1·06
	—	—	1·22	—	—	—	1·08	—	—	—	0·95	—
	—	—	1·02	—	—	—	1·22	—	—	—	0·94	—
Average	1·18	1·25	1·11	1·08	1·34	1·40	1·31	1·34	1·04	1·06	1·00	1·01

Stage of growth. Lugg [1938, 2] could not find any association between the composition of extracted protein and the age of the leaves, but the results of this investigation show that stage of growth does affect both cystine and methionine. In general, the young and grazed samples contain less cystine and methionine than the older samples, the maximum figures being reached at about the stage of flowering or heading. This holds good for all four species with the single exception that the cystine-N in a sample of cocksfoot at flowering stage taken in July is the lowest figure for all the cocksfoot samples.

When the actual dates of sampling were considered it was obvious that the figures for clover reached a maximum in July and then decreased, whereas with the grasses the maximum in July was followed by a decline in August or September and another rise in September or October. This was the case for both cystine and methionine and this difference in seasonal variations between the clover and grasses may be explained by the fact that the growing period of the former is somewhat restricted, whereas the grasses continue to grow throughout the season.

The numbers of samples in the various age groups are too small to permit many positive assertions, but the figures have been submitted to statistical analysis and, where the number of degrees of freedom permitted, the significance of the differences of means has been tested by the usual t-technique—t being taken at 2·5. The laboratory standard errors (amounting to about $\pm 2{\cdot}5\%$ for cystine and $\pm 3{\cdot}5\%$ for methionine by homocysteine) were duly considered and significant differences were found to exist in the following cases:

Cystine. White clover. Flowering v. young or aftermath or grazed; old v. aftermath or grazed.

Cocksfoot. Old v. aftermath or grazed.

Rye-grass. Aftermath v. flowering or old.

Timothy. No significant differences.

Methionine. White clover. Young v. flowering; aftermath v. flowering or old.

Cocksfoot. Old v. aftermath.

Rye-grass and timothy. No significant differences emerge.

There seems to be little doubt, therefore, that both cystine and methionine rise to significant maxima when the plants have reached or passed the heading stage and that they are present in smaller amounts in the aftermath or grazed samples. This is supported by the results in Table 4, which show how the sulphur, other than that present in cystine or methionine, is almost invariably lower in the flowering or old samples than in the others. It is also of interest to note that there is a fairly strong relation between cystine and methionine, the latter on an average being about nine-tenths of the former.

Table 4. *Percentage distribution of S in protein preparations (methionine by homocysteine)*

Nature of sample	White clover			Cocksfoot			Rye-grass			Timothy		
	Cyst.	Meth.	Rest	Cyst.	Meth.	Rest	Cyst.	Meth.	Rest	Cyst.	Meth.	Rest
Young	43·4	38·3	18·3	41·9	38·2	19·9	36·3	36·3	27·4	38·8	35·2	26·0
	36·6	34·0	29·4	—	—	—	—	—	—	—	—	—
	41·1	37·4	21·5	—	—	—	—	—	—	—	—	—
Flowering or heading	47·9	41·4	10·7	45·4	37·2	17·4	47·1	43·0	9·9	49·7	40·0	10·4
	45·6	41·4	13·0	40·5	43·6	16·0	46·8	42·8	10·4	43·8	43·1	13·1
	47·6	38·8	13·7	43·3	39·4	17·4	47·7	41·7	10·6	—	—	—
	—	—	—	54·7	36·7	8·6	51·8	40·6	7·6	—	—	—
Old	48·9	40·9	10·1	48·9	40·9	10·2	47·7	43·0	9·3	46·7	44·8	8·6
	46·5	43·0	10·5	45·9	40·0	14·1	47·7	43·4	9·0	—	—	—
	46·6	41·1	12·3	47·2	40·3	12·5	—	—	—	—	—	—
Aftermath	37·3	33·3	29·4	45·9	33·1	21·0	39·4	43·1	17·6	43·4	40·5	16·1
	39·3	35·0	25·6	48·1	41·5	10·4	43·2	41·3	15·4	39·3	35·5	25·2
	—	—	—	44·4	35·6	20·0	40·5	36·8	22·8	—	—	—
	—	—	—	49·7	39·7	10·6	41·2	36·2	22·6	—	—	—
	—	—	—	42·5	39·9	17·6	44·1	40·2	15·7	—	—	—
	—	—	—	49·0	39·2	11·9	—	—	—	—	—	—
Grazed	40·7	31·6	27·7	41·4	37·8	20·8	43·1	39·1	17·8	37·4	42·6	20·0
	40·5	39·0	20·5	41·5	38·2	20·3	42·6	38·9	18·4	44·7	37·9	17·5
	—	—	—	—	—	—	50·9	39·6	9·5	—	—	—
	—	—	—	—	—	—	42·7	39·4	17·9	—	—	—
Average	43·2	38·1	18·7	45·6	38·8	15·5	44·6	40·3	15·1	43·0	40·0	16·9

There is an association between the total amino-N and the S-containing amino-acids in the proteins. The actual correlation coefficient for cystine + methionine and total amino-acids is +0·45, which is quite significant for sixty-three samples.

Environment. Although a substantial amount of evidence is available to show that the mineral matter in herbage is determined to a large degree by soil conditions, it is generally recognized that the composition of the organic matter is affected mainly by stage of growth. It was impossible to obtain samples at exactly the same stage of growth from the different sites in order to compare the influence of local soil and climatic conditions; from the data collected, however, any effects of locality were not apparent for any of the species concerned.

As has been pointed out elsewhere [Smith & Wang, 1941], grazing exerts a very important influence on the composition of grass, both the carotene and total N being considerably increased. This investigation shows that grass kept short by grazing contains cystine and methionine in amounts similar to those for young grass or aftermath. The effects of manuring were observed in only a few

cases and were rather irregular and relatively unimportant. Lime produced a slight increase in the total amino-acids of the proteins of both cocksfoot and rye-grass, but the cystine and methionine were not altered to a significant extent. Slag had opposite effects on the two species. The effect of applying a dressing of ammonium sulphate at the rate of 1 cwt. per acre to rye-grass, 3 weeks before cutting, was to reduce the percentage of N as amino-N in the hydrolysed protein from 65 to 57 and to effect smaller reductions in the contents of cystine and methionine.

Summary

Protein preparations have been made from sixty-three samples of *Trifolium repens*, *Dactylis glomerata*, *Lolium perenne* and *Phleum pratense*, by mincing and extraction with a buffer solution (*p*H 9·2), precipitation at *p*H 4·5, washing with alcohol and ether and finally drying in a vacuum.

The proteins contained 15–43 % of the total N in the plants. The amount of N in the protein was on the average about 11 %, being slightly higher in the preparations from the clover than in those from the grasses. The clover protein also contained a rather greater proportion of amino-N (67 %) after hydrolysis than the grass proteins (60–63 %).

The distribution of S as cystine, methionine and sulphate was estimated by hydrolysis with HI followed by determination of the cysteine and homocysteine in the digest and of methyl iodide, sulphide and sulphite in the absorption train. The values for methionine were much higher by the methyl iodide method than by the homocysteine method; expressed as percentage of N in the protein the averages were 1·35 and 1·03 % respectively. The amounts of cystine (1·16 %) and methionine were essentially the same for all four species but they were significantly greater in samples which had reached or passed flowering stage than in samples of young grass or aftermath or grass which had been kept short by grazing. The reverse was the case for sulphate-S which accounted for from about 8 to 29 % of the total S measured. Neither site nor manuring, with the exception of a late dressing of N, seemed to influence the results.

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41. THE PROTEINS OF *HEVEA BRASILIENSIS*
2. ANALYSIS OF A PRODUCT ISOLATED FROM CREPE RUBBER

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It has long been suspected that proteins played some part in the chemistry and technology of latex and raw rubber, but it is only recently that evidence has been obtained as to their exact function. It is now known [Bondy & Freundlich, 1938; Kemp & Straitiff, 1940] that the proteins in latex are the controlling factors in its stability. Thus, the coagulation point of latex corresponds to the isoelectric point of the proteins in solution; dialysed latex is not readily coagulated by acid unless a small concentration of salt is added, a property which is also common to the proteins. When latex is concentrated in the factory by centrifugal methods it is rendered much more unstable, presumably because it has lost a large amount of the protein which acted as a stabilizing agent.

Knowledge of the influence of proteins on the properties of raw rubber has been much more vague. It is known, however [Davis & Blake, 1937], that they do exert a considerable influence on the vulcanizing properties of rubber and that the water absorption of raw rubber depends on the amount of protein present [Skinner & Drakeley, 1931]. More recently Bloomfield & Farmer [1940] have shown that when crepe rubber is fractionated a series of N-free fractions are obtained which are soluble in the usual rubber solvents. The end-fraction which contains the bulk of the N is, however, insoluble. If, however, N-free rubber (prepared from trypsin-digested latex) is used as the starting material then all the fractions are soluble. Gee & Treloar [1940], using the fractions of Bloomfield & Farmer, have shown that the N-free fractions fall into a definite molecular series, whereas the fractions which contain N are radically different in behaviour.

Tristram [1940] described the isolation and analysis of a protein from dried fresh latex films. It was not known what relationship this product bore to the total protein of latex or to the protein which is associated with the hydrocarbon in raw rubber. De Vries [1924] expressed the opinion that latex contained two proteins, only one of which was precipitated with the rubber, but advanced no evidence to prove this view. Bondy & Freundlich [1938] isolated two protein fractions from ammonia-preserved latex and showed that both had isoelectric points within the precipitation range of latex but made no statement as to the presence of a third protein. Kemp & Straitiff [1940] repeating the work of Bondy & Freundlich claimed the presence of a third protein which was not coagulated with the hydrocarbon. Thus, if the findings of Kemp & Straitiff are correct, latex contains three proteins A, B and C, of which A and B are precipitated with the rubber. The preparation extracted from dried fresh latex films [Tristram, 1940] by borate may therefore be a mixture of A, B and C or, more probably, a sample rich in protein C. Unfortunately, none of the above workers characterized his preparation by analytical methods, so that it cannot be said definitely that A, B

and C were separate entities. The present work was accordingly carried out to determine the relationship of this borate-extracted protein to the total protein of latex and to the protein found in raw rubber.

Experimental

The material chosen as the source of protein was the common commercial form, crepe rubber. The method of extraction first tried was that used previously [Tristram, 1940], but no protein was isolated. This suggests either that the protein in crepe rubber is somewhat denatured or that the protein is intimately associated with the rubber hydrocarbon. The latter seems to be more probable in view of the findings of Bloomfield & Farmer [1940] and of Gee & Treloar [1940].

The method of isolation adopted ultimately was based on that of Midgeley *et al.* [1937], since it depended on the dissolution of the rubber from the protein. The method has been modified in accordance with recent work in the above laboratories [Bloomfield & Farmer, 1940].

Method of isolation

500 g. shredded crepe rubber were extracted with acetone, in a drip extractor, for 24 hr. in an atmosphere of N_2. The residue was dried *in vacuo* and then extracted continuously with light petroleum under N_2 in an apparatus designed by Dr Michael [see Bloomfield & Farmer, 1940]. This procedure removed the '*sol*' rubber (*ca.* 50 % total), which was virtually nitrogen-free (0·05–0·02 %). The remainder, '*gel*' rubber, was coagulated with acetone, dried *in vacuo* and exhaustively extracted with xylene at 60–70°; during which time the mixture was disturbed as little as possible, the organic solvent being replaced by decantation every 24 hr. The residue was next refluxed *in vacuo* with xylene and the insoluble material removed in the centrifuge. The final residue, a gelatinous mass, was extracted successively with xylene, benzene and toluene and then dried *in vacuo*. The dry material was brittle and fibrous; it contained 34–35 % rubber, 8·13 % ash and 8·9 % N; the N content, ash- and rubber-free, was 14·6 %. So far it has not been found possible to separate the protein and rubber otherwise than by methods which involve breakdown of the protein, i.e. acid or alkaline hydrolysis. This finding is in keeping with that of Bloomfield & Farmer [1940] discussed above.

Analysis of product

The yield of protein, after correcting for the rubber content, was 2·3 g. or 0·46 % of the crepe. Owing to lack of material the analysis (Table 1, 2) could not be made so extensive as those reported previously (Table 1, 1), but it was

Table 1

(Results are given as percentage of total N)

Method:	Dried latex 1 Borate buffer	Crepe rubber 2 Organic extraction
Yield g. %	0·1–0·15	0·46
Total N (ash- and rubber-free)	14·95	14·65
Amide-N	7·07	7·18
Tyrosine-N	3·3	3·1
Tryptophan-N	1·2	0·0–0·7
Arginine-N	12·9	13·0
Lysine-N	6·4	6·8
Glutamic acid-N	7·65	Not less than 1·8
Aspartic acid-N	6·5	Not less than 4·1

considered that values for ammonia, tyrosine, tryptophan, lysine and arginine would be sufficient for the purpose of comparison. In addition, the dicarboxylic acids have been investigated qualitatively to test the validity of the claim of Midgeley *et al.* [1937] that glutamic acid is absent from the protein of raw rubber.

Discussion

The results show clearly that the two products have the same contents of ammonia, tyrosine, arginine and lysine, and in view of this agreement it is suggested that either there is only one protein in latex or that there is a mixture of proteins of which preparation 1 is a representative sample. The protein from crepe rubber was different in tryptophan content. One sample contained no tryptophan and a second, prepared from a new sample of crepe, contained 0·7% of tryptophan-N. It is known that tryptophan is a labile amino-acid, being removed from the protein molecule extremely easily by digestion: it is more probable, however, that in the present case some change in the specificity of the amino-acid has occurred in the intact protein molecule during the manufacture of the crepe rubber from latex. ($NaHSO_3$ is often added to latex before coagulation with formic acid and after being coagulated and washed on the rollers the crepe is dried for 2 to 3 weeks in a tropical atmosphere ($30° \pm$).) The loss of tryptophan is being investigated and the preliminary results indicate that $NaHSO_3$ does affect this amino-acid. Furthermore, proteinaceous material has been isolated from crepe rubber which had been oxidized in the presence of acetic acid [Bloomfield—unpublished experiments] and it has been found that this material contains only 0·2–0·3% of tryptophan-N whereas the tyrosine-N is 2·8–3·1%. These results suggest that the change in the specificity of tryptophan does take place during the manufacture of crepe rubber.

If the above suggestion is correct it follows that this protein or mixture of proteins is precipitated, more or less completely, on the coagulation of latex and is thus present in crepe rubber. Such a finding would be in keeping with the evidence of Bondy & Freundlich [1938] referred to above, but is in contrast to the ideas of De Vries [1924] and Kemp & Straitiff [1940]. It has been observed on numerous occasions [cf. Altman, 1939; Whitby & Greenberg (unpublished)] that some protein does, indeed, remain in the serum after coagulation of the latex. These workers, however, used serum prepared under factory conditions, and it is probable that some of the protein escaped precipitation since the *p*H would not be rigidly controlled. It is also possible that there are present in latex small amounts of proteinaceous material, probably a degradation product of the native proteins, which remains soluble at the coagulation point of the latex.

That the main bulk of the protein is precipitated is seen from the following experiment. A sample of latex preserved with sodium pentachlorophenate was found to contain 0·136% protein-N. The dry rubber content was 38·0%, so that the calculated N content of the rubber, assuming that the whole of the protein is precipitated, should be 0·37%. A sample of the latex was then coagulated and the rubber creped under semi-factory conditions. The N content of the crepe was 0·37%.

The analytical results given in Table 1 are not in complete agreement with the qualitative experiments of Midgeley *et al.* [1937], who obtained no evidence for the presence of cystine, tyrosine and glutamic acid in the proteins of raw rubber. The absence of tyrosine has also been suggested by Kemp & Straitiff [1940], who reported that the proteins of latex gave a negative Millon's test. It would appear that the analytical methods used by both sets of workers were inadequate for the purpose.

SUMMARY

A protein (2) has been isolated from crepe rubber and analysed for amide-N, tyrosine, tryptophan and the basic amino-acids. The dicarboxylic acids have been shown to be present.

The results indicate that preparation 2 is of the same composition as preparation 1 [Tristram, 1940].

It is now suggested that preparation 1 is a representative fraction of the total protein of latex.

Loss of tryptophan has been observed from the protein found in crepe rubber and a reason for this loss has been suggested.

The author wishes to thank Prof. A. C. Chibnall, who was responsible for directing the course of the research, and whose advice and criticism have proved invaluable.

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42. THE SEPARATION OF AMINO-ACIDS BY MEANS OF THEIR COPPER SALTS

3. THE HYDROLYSIS OF GLIADIN. AN INVESTIGATION OF THE DICARBOXYLATE FRACTION, INCLUDING THE ISOLATION OF *r*-GLUTAMIC ACID AS AN HYDROLYSIS PRODUCT

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The study of the hydrolysis of gliadin described in this paper was started during the tenure of an Alexander Brown Coxe Research Fellowship at Yale University during the year 1931–32. It is gradually being completed with a view to obtaining as complete a picture as possible of the disposition of the amino-acids in their separation by means of their copper salts.

In the original paper [Town, 1928] three fractions were obtained by separating the copper salts of the amino-acids into:

1. Those soluble in methyl alcohol.
2. Those soluble in water, but insoluble in methyl alcohol.
3. Those insoluble in water.

In a later paper [Town, 1936] the methyl alcohol-soluble copper salt fraction was further subdivided into (*a*) a part precipitated by alcoholic $CdCl_2$ and (*b*) the filtrate. The examination of the $CdCl_2$ precipitate, which contains the proline, was fully reported.

Modifications were introduced into this hydrolysis as a result of experience gained in previous hydrolyses. Important technical improvements were as follows.

(*a*) Copper hydroxide was used in place of the basic copper carbonate formerly employed. This substance is much more finely divided and more reactive, and when the amino-acids are evaporated with copper hydroxide to form their copper salts, the calculated quantity is sufficient, no excess being required.

(*b*) Earlier work had shown that unless the ammonia is first removed, cuprammonium compounds are formed during copper-salt formation and these interfere greatly with the true solubility of the copper salts of the amino-acids in the solvents employed, namely methyl alcohol and water.

(*c*) The dicarboxylates also interfered in the examination of the methyl alcohol-soluble copper salts [Town, 1936], for it was then found that 2·06 % of the glutamic acid, which should be present in the water-soluble copper salts, was isolated from the methyl alcohol-soluble copper salt fraction. This interfered with the isolation of proline.

The fact that both ammonia and the dicarboxylic amino-acids interfered during the copper salt separation suggested their prior removal. The dicarboxylates were first precipitated by the usual modification of Foreman's method

[Foreman, 1914], in which baryta takes the place of lime. The filtrate containing alcohol was then concentrated under reduced pressure to remove both the alcohol and the ammonia. The solution left was then freed from any excess baryta and converted into copper salts in the usual manner by evaporating with $Cu(OH)_2$.

The exhaustive examination of the amino-acids precipitated according to Foreman's method or by the common modification of using baryta in place of lime is of prime importance to protein chemists, for conflicting statements have been issued as to the extent that other amino-acids are precipitated as Ca or Ba salts insoluble in alcohol. The results of the present investigation do not seem to bear out any suggestion that other amino-acids are precipitated by the usual procedure. Thus 96·4 % of the nitrogen has been accounted for and the only amino-acids isolated from the dicarboxylate fraction were glutamic acid, aspartic acid and serine. The nitrogen distribution in the fraction is fully indicated in the scheme on p. 431.

The Ba salts of the dicarboxylic acids after precipitation were decomposed and the solution of the amino-acids, containing 27 % of the total N, concentrated and saturated with HCl gas. Five crops of glutamic acid hydrochloride were isolated, the first four crops being homogeneous and having M.P. above 202°. Details of their properties are given in Table 1. It will be noted that 87·5 % of the glutamic acid was isolated in the first crop. The first four crops yielded 254·5 g. of *l*(+)-glutamic acid hydrochloride in practically pure condition, whereas the 5th crop gave 3·0 g. of a fraction with a very low positive rotation and a low melting point. This was at first thought to be a very impure fraction, until it was found much later on to yield an anhydrous 3:5-dinitrobenzoyl derivative, M.P. 204° (51 % yield), whereas *l*(+)-glutamic acid gives rise to a similar derivative, M.P. 104° (40 % yield), crystallizing with one molecule of water.

A complete investigation of the mother liquor from the 5th crop increases the yield of this amino-acid with the 3:5-dinitrobenzoyl derivative, M.P. 204°, to 1·93 % of the dry protein or 1·06 % of the total N. A chemical investigation described below has shown it also to be a glutamic acid, and evidence is adduced which would lead to the belief that it is racemic glutamic acid (as distinct from the *dl* mixture), and that it is an hydrolysis product and not an artefact. The ratio of racemic to *l*(+)-glutamic acid is 1/19·6.

This isolation of racemized glutamic acid from a normal protein is of great interest in view of the discussion which has recently taken place between Kögl & Erxleben [1939, 1, 2 & 3] and Chibnall *et al.* [1939; 1940], concerning the glutamic acid of normal and malignant tissue proteins. The former authors asserted that the glutamic acid isolated from malignant tissue protein was partially racemized and they suggested that it was a characteristic of such proteins. Chibnall found that tumour proteins contained the bulk of their glutamic acid in the ordinary active form and he thought that any racemization was incidental to the methods employed in the isolation of the glutamic acid and had no special significance with respect to cancer tissue.

The isolation of racemic (as distinct from *dl*) glutamic acid from gliadin shows that a normal protein may contain some of its glutamic acid in the inactive racemic form and it may be a general characteristic of proteins. It would appear not to be a criterion of malignancy in living tissue. However, the suggestion that proteins may contain some portion of their constituent amino-acids in an optically inactive form is, as far as the author is aware, a new conception, and it will be interesting to see if in the future other amino-acids are

shown to exist in the same state. The author contributed to a symposium which recently appeared on the subject of the glutamic acid of proteins [Town, 1940].

The total yield of glutamic acid isolated from the sample of gliadin used is 40·4 % of the dry protein or 22·1 % of the total N; these figures are made up as follows:

	% of dry protein		N isolated / total N	
l(+)-glutamic acid	37·81		20·7	
dl-(mixture)glutamic acid	0·69	2·62	0·38	1·44
r-(compound)glutamic acid	1·93		1·06	
	40·43		22·14	

It is interesting to note that the M.P. of *r*-glutamic acid hydrochloride is 194°, for divergent values for the M.P. of glutamic acid hydrochloride, are quoted in the literature. The following values for the M.P. of the different varieties of this substance have been found during the course of this work: *l*(+)-glutamic acid hydrochloride, 207°; *dl*-, 213°; *r*-, 194°.

The minute examination of the mother liquor from the precipitation of the glutamic acid hydrochloride was originally undertaken with a view to isolating hydroxyglutamic acid, which has been said to be present in gliadin. The isolation of this acid has proved difficult to many workers and although it was first isolated by Dakin as long ago as [1918] from caseinogen, its occurrence as an hydrolysis product of proteins cannot yet be said to have been substantiated. The mother liquors from the precipitation of glutamic acid hydrochloride were first re-precipitated with baryta and alcohol to ensure that only dicarboxylates were present, and then the free amino-acids were converted into copper salts to separate any aspartic acid as its insoluble copper salt. The soluble copper salts were freed from copper, and the free amino-acids treated with silver sulphate and baryta to precipitate any Ag hydroxyglutamate as recommended by Dakin [1918]. The treatment that the precipitate and filtrate from this Ag precipitation underwent is clearly indicated in Diagram 2 and it can be seen that no separation has been effected, for both precipitate and filtrate were found to contain *r*-glutamic acid. No trace of hydroxyglutamic acid was detected during the course of this work, although special care has been taken to look for it, and it is the author's considered opinion that it is not present in gliadin. It is interesting to note that 1·1 g. of serine have been isolated from the final fractions, and it seems probable that a mixture of say *r*-glutamic acid and serine may have been mistaken for this acid.

Another important point which this investigation has raised is the identity of copper aspartate. The copper aspartate isolated during this analysis appeared to be homogeneous, but on decomposition and fractional crystallization of the free amino-acid, it was shown to be a mixture of aspartic acid and *r*-glutamic acid in approximately equal proportions. Whether this is a general behaviour or something peculiar to this analysis has yet to be decided. The author has examined three laboratory samples of aspartic acid and all of them have been found to contain some proportion of the *r*-glutamic acid (the smallest proportion being approximately 4 %). Unfortunately their previous history is unknown. It might be mentioned that although aspartic acid is said to melt at 270°, it has been found in this laboratory that the only sample of aspartic acid which melted at all also contained *r*-glutamic acid and had M.P. 273° (indefinite). All pure samples of this acid prepared in this laboratory have M.P. >320°, and have not yet been made to melt.

Experimental

Preparation of the gliadin. A brief description is given owing to enquiries as to the method of preparation. 5 kg. of a commercial gluten preparation obtained from 'The Battle Creek Food Co.', Battle Creek, Michigan, were extracted with 25 l. and then with 10 l. of 73 % alcohol, each time being left overnight. The alcohol extract was poured off and the gluten residue wrapped in calico and squeezed in a hydraulic press. The extract was a clear yellow liquid (29 l.) and it was concentrated at 45° under reduced pressure to 1/3 volume. It was poured into ice-cold water and the gliadin precipitated as a thick viscous material which was transferred to a large basin and treated with three changes of dry acetone. The gliadin fell rapidly away to a fine powder and any lumps were passed with acetone through a hand mincing machine. The powder was left with dry acetone for several hours, filtered on a Büchner funnel, and spread on brown paper to dry.

Properties. The preparation is a fine white powder and dissolves in 73 % alcohol to a clear colourless solution. It contains 15·2 % N, 10·22 % moisture and 1·08 % fat (ether-soluble material). The N on a moisture and fat free basis is 17·40 %. The specific rotation using a 7 % solution of the air-dried specimen was $-79·5°$, which is equivalent to $-88·8°$ for the anhydrous protein.

Hydrolysis

600 g. of the air-dry protein (532 g. anhydrous protein) were boiled under reflux for 36 hr. with 30 % w/v H_2SO_4. The total N in the hydrolysed protein was 91·6 g. The hydrolysed solution (3200 ml.) was diluted with 5100 ml. water and the humin material filtered off (8·6 g.; N, 0·3 g.). The filtrate was treated with hot aqueous baryta until just acid to Congo red, the $BaSO_4$ being filtered and washed with acidified boiling water (wt. $BaSO_4$, 2214 g.; N, 3·02 g.). The filtrate and washings were concentrated to 2870 ml. (N, 88·0 g.) and 786 g. of baryta in hot water added. To the resulting 5 l. of solution, 15 l. 95 % alcohol were added with constant stirring and the powdery but bulky precipitate of barium dicarboxylate allowed to settle overnight before filtration. The bulk of the supernatant alcohol, which was alkaline to litmus, was siphoned off and concentrated under reduced pressure (bath at 50°) to remove the ammonia before converting into copper salts. The Ba-dicarboxylate precipitate was finally filtered and washed once with 60 % alcohol.

The dicarboxylate precipitate. The precipitate of Ba salts was dissolved in dil. HCl (vol. solution 3 l.) and the Ba removed quantitatively; the $BaSO_4$ precipitate was washed twice by boiling with 800 ml. 5 % HCl (wt. $BaSO_4$, 740 g.; N, 0·86 g.). The amino-acid solution after concentration had volume 2300 ml. (N, 24·61 g.; 26·97 % of total N (91·62 g.)) and the concentration was continued until glutamic acid hydrochloride commenced to crystallize (vol. 1500 ml.). The solution was now cooled in ice and saturated with HCl gas; a large crystalline precipitate formed. It was filtered off, dissolved in 1200 ml. water and reprecipitated by saturation with HCl gas. The precipitate was filtered, and weighed 225 g. (1st crop).

The mother liquors were concentrated to 400 ml. and again saturated with HCl gas, giving a second crop of crystals which on recrystallization weighed 15 g. There was similarly obtained a 3rd crop (wt. 7·5 g.); the solution was now very dark brown and so it was boiled with norite (0·5 g.), filtered, concentrated to 100 ml. and again treated with HCl gas (4th crop, wt. 7·0 g.); on saturating the mother liquor (30 ml.) with HCl a 5th and final crop of hydrochloride was obtained (wt. 3·0 g.). The properties of these five crops of hydrochlorides are shown in Table 1, while the treatment of the mother liquor is described later.

Table 1

Crop	Wt. of hydrochloride g.	M.P. of hydrochloride °C.	Wt. as free glutamic acid g.	% N	% Cl	Test for NH_3	$[\alpha]_D$ ($c=4$ in H_2O)	Yield N/total N %	Yield Amino-acid Protein %
1	225	207	181	7·55	19·40	—	+25·0	18·5	34·0
2	15	203	12·1	7·40	19·45	—	+20·8	1·1	2·3
3	7·5	202	6·0	7·43	19·5	—	+19·1	0·6	1·1
4	7·0	202	5·6	7·40	19·4	—	+24·0	0·6	1·1
5	3·0	175	2·4	7·45	19·3	Just +ve	+ 5·8	0·25	0·5
Total	257·5		207·1					21·05	39·0
							Excluding 5	20·8	38·5
Properties of glutamic acid hydrochloride:									
	Softens M.P.	203 207		7·63	19·34		+25·0		

Although the information in Table 1 was collected over a long time it has been put together without further explanation, although it should be pointed out that the rotations have only recently been determined.

The yield of glutamic acid expressed in terms of *l*(+)-, *d*(−)- and *dl*-mixture as calculated from the rotations is as follows:

	% of protein			
	I		II	
	l(+)-acid	*dl*(mixture)-acid	*l*(+)-acid	*d*(−)-acid
Crop 1	34·0	0·0	34·0	0·0
Crop 2	1·91	0·39	2·11	0·20
Crop 3	0·84	0·26	0·97	0·13
Crop 4	1·06	0·04	1·08	0·02
	37·81	0·69	38·16	0·35

To this has to be added the amount of glutamic acid isolated from crop 5 and later fractions which is thought to be *r*-glutamic acid, namely 1·93 % of the dry protein. This makes a total yield of 40·4 %.

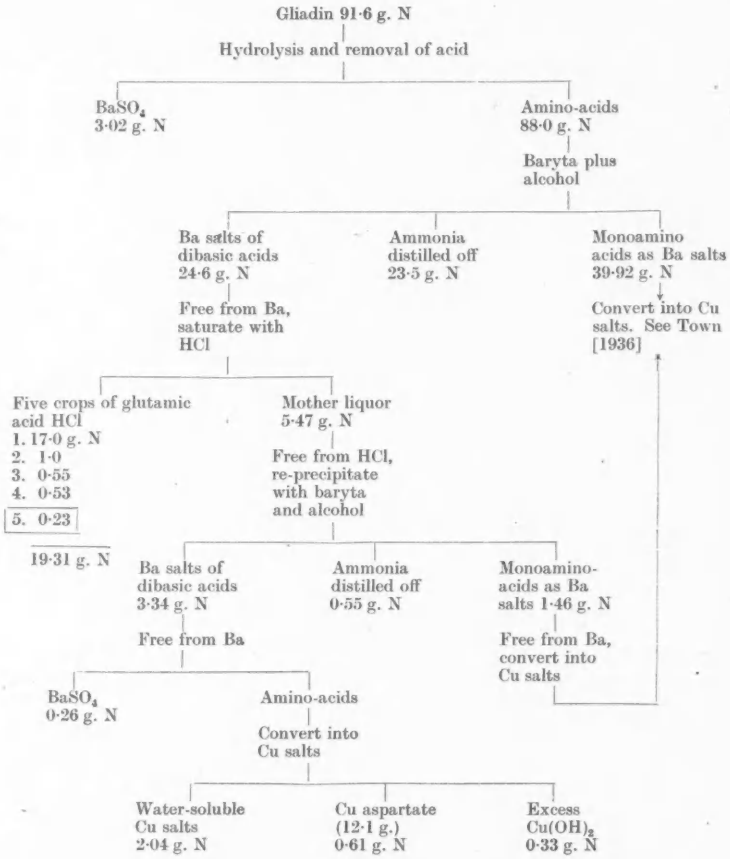
Examination of mother liquor from the precipitation of glutamic acid hydrochloride

The mother liquor from the 5th crop was evaporated to a syrup and then diluted to one litre; 25 ml. 12 *N* H_2SO_4; were added and the HCl removed by adding Ag_2O to slight excess (test with baryta), excess Ag being removed with H_2S. The precipitates were washed by boiling twice with water (AgCl, wt. 58·6 g.; N, 0·01 g.: Ag_2S, wt. 1·3 g.; N, negligible); the filtrate and washings were concentrated to 710 ml. (N, 5·47 g.). It was decided at this stage to reprecipitate the dicarboxylates, if any, with the group reagent baryta and alcohol: 220 g. $Ba(OH)_2$, $8H_2O$ were added and the combined volume was 800 ml.; 3 vol. 95 % alcohol were added and the granular precipitate allowed to settle, filtered and washed. Weight of precipitate 180 g. (N, 3·34 g.). The filtrate (3660 ml.; N, 2·02 g.) was evaporated under reduced pressure and the N in the aqueous residue determined (vol. 495 ml.; N, 1·46 g.). This indicates that 2·02–1·46, or 0·55 g., N was still present as ammonia and had distilled over. The distillate gave strongly positive tests for NH_3.

The aqueous residue (N, 1·46 g.) was converted into copper salts and mixed with the main bulk of copper salts of the monoamino-acids obtained after the

original precipitation of the dicarboxylates. This action was later regretted, as it would have been interesting to see what amino-acids were precipitated with the dicarboxylates at their first precipitation, but one is in great danger of needlessly multiplying fractions. Before proceeding further, a schematic diagram will show the exact stage reached in the separation (Diagram 1).

Diagram 1


Gliadin 91·6 g. N
Hydrolysis and removal of acid
$BaSO_4$ 3·02 g. N
Amino-acids 88·0 g. N
Baryta plus alcohol
Ba salts of dibasic acids 24·6 g. N
Ammonia distilled off 23·5 g. N
Monoamino acids as Ba salts 39·92 g. N
Free from Ba, saturate with HCl
Convert into Cu salts. See Town [1936]
Five crops of glutamic acid HCl
1. 17·0 g. N
2. 1·0
3. 0·55
4. 0·53
5. 0·23
19·31 g. N
Mother liquor 5·47 g. N
Free from HCl, re-precipitate with baryta and alcohol
Ba salts of dibasic acids 3·34 g. N
Ammonia distilled off 0·55 g. N
Monoamino-acids as Ba salts 1·46 g. N
Free from Ba
Free from Ba, convert into Cu salts
$BaSO_4$ 0·26 g. N
Amino-acids
Convert into Cu salts
Water-soluble Cu salts 2·04 g. N
Cu aspartate (12·1 g.) 0·61 g. N
Excess $Cu(OH)_2$ 0·33 g. N

The precipitated Ba dicarboxylates (N, 3·34 g.) were dissolved in one litre of water and freed from Ba (wt. $BaSO_4$, 120 g.; N, 0·26 g.). The amino-acids were converted into copper salts by boiling with 25 g. $Cu(OH)_2$, filtered from excess $Cu(OH)_2$ (wt. 12 g.; N, 0·33 g. after boiling three times with water), and left in the ice-chest to cool; a mass of light blue feathery crystals of copper aspartate separated (wt. 12·1 g.; N, 0·61 g.; this represents 1·09 % of the dry protein or 0·66 % of total N; loss in weight on drying is 24·6 %; however, see later.)

The filtrate from the copper aspartate was freed from Cu (wt. CuS, 11 g.; N, 0·016 g.) and the solution concentrated to 1050 ml. (N, 2·04 g.). This solution was now treated as described by Dakin [1918] for the isolation of hydroxyglutamic acid. It was treated with precipitated AgOH and a little sulphuric acid (just sufficient to keep it acid to Congo red) until excess of Ag was present (brown precipitate with baryta). Baryta water was now added until *p*H 7 was reached and the white precipitate filtered off; on bringing the filtrate to *p*H 10 a small black precipitate formed which was also filtered off. The final filtrate had vol. 1430 ml. (N, 1·30 g.) and so the quantity of N precipitated should be 0·74 g., i.e. 36·3 % of the total. All fractions were freed from Ag (Ag_2S from precipitates 27·3 g.; N, 0·01 g.: Ag_2S from filtrate 13 g.; N, 0·006 g.) and the solutions concentrated on the water bath (65°) under reduced pressure.

Ag precipitate. On evaporation, tetrahedral crystals separated rather like glutamic acid, but the solution was evaporated to dryness and extracted by grinding with glacial acetic acid and left overnight. Only a small portion dissolved; the insoluble material being centrifuged off and again treated with acetic acid for 12 hr. Finally it was dissolved in 2 ml. H_2O and saturated with HCl gas (wt. of precipitate 1·7 g.). At first this was thought to be ordinary glutamic acid hydrochloride and was not examined. Later, however, on attempting to prepare the 3:5-dinitrobenzoyl derivative it was found to yield mainly a derivative, M.P. 204°, together with a trace of the normal glutamic acid derivative, M.P. 104°. Yield: 0·64 g. amino-acid hydrochloride gave 0·31 g. derivative, M.P. 204° (26·1 % of theory) and 0·02 g. derivative, M.P. 104° (1·5 %).

The material soluble in acetic acid was treated with methyl alcohol to cause granulation, dried and again treated with acetic acid. A trace (0·16 g.) was insoluble. The solution was concentrated to 30 ml. and poured into one litre of acetone when the material granulated: wt. 2·05 g.; N, 0·19 g.

Filtrate from Ag precipitation. As evaporation proceeded crystals separated in long needles which were identified as tyrosine (wt. 0·15 g.; N, 7·5 %; Millon positive); later a further crop of tetrahedral crystals separated (wt. 1·5 g., M.P. 197°), and finally a 3rd crop, also tetrahedra, containing a few coarse needles (wt. after crystallizing 0·8 g., M.P. 187–188°). Later it was found that both these fractions yielded 3:5-dinitrobenzoyl derivatives of M.P. 204° identical with the material obtained from the Ag precipitate. No further crops of crystals could be obtained and the syrupy residue was treated with glacial acetic acid, when all but 0·3 g. dissolved. The solution was evaporated *in vacuo* over NaOH and redissolved in glacial acetic acid, but 0·4 g. was insoluble. This 0·7 g. material was also found to yield a 3:5-dinitrobenzoyl derivative, M.P. 202°. The acetic acid solution was concentrated and granulated by pouring into one litre of acetone. The bulk of the material was insoluble (N 0·68 g.), but the acetone solution was slightly coloured; it was mixed with that from the Ag precipitate and was found to contain 0·24 g. N.

As the precipitation with silver and baryta according to the method of Dakin had apparently effected no separation the fractions were recombined to give (*a*) material insoluble in acetone containing 0·68 + 0·19 or 0·87 g. N; (*b*) material soluble in acetone (0·24 g. N). On evaporation both solutions gave syrupy residues and, after many experiments, these were dissolved separately in 50 % methyl alcohol and precipitated by adding a 20 % solution of mercuric acetate in 50 % methyl alcohol.

Acetone-soluble. N precipitated 0·10 g. (41·5 %); this was not examined further as it is probably diketopiperazine material.

Acetone-insoluble. Volume 310 ml. N precipitated 0·66 g. (76 % of total).

The mercury precipitate was decomposed and the solution on evaporation gave a syrup which on stirring suddenly solidified to a waxy solid; this became powdery on treatment with methyl alcohol (wt. 3·58 g.; N, 11·1 %; amino-N 97·5 %, M.P. 165°, softens 140°, no charring). The equiv. wt. by titrating in alcohol solution was 104; it formed a blue copper salt which did not crystallize. A further batch of this crystalline material (wt. 1·4 g.; N, 11·0 %) was obtained by evaporation and treatment with MeOH.

As this material seemed to be a mixture it was decided to mix the fractions and reprecipitate them with mercuric acetate. The N content of mixed crops was 0·54 g., of which 0·46 g. (87 %) was precipitated. The Hg precipitate was decomposed and the amino-acid solution evaporated under reduced pressure. It was difficult to work with, but on adding methyl alcohol till the solution was turbid and keeping, a quantity of hard gritty crystals formed (wt. 0·51 g.; N, 9·73 %; M.P. 188°), crystallizing in rectangular prisms. This was also later found to yield a 3:5-dinitrobenzoyl derivative M.P. 202°. The mother liquor on evaporation and retreatment with methyl alcohol yielded more crystals, also gritty to the touch (wt. 0·46 g.; N, 9·65 %). Its 3:5-dinitrobenzoyl derivative had M.P. 202°.

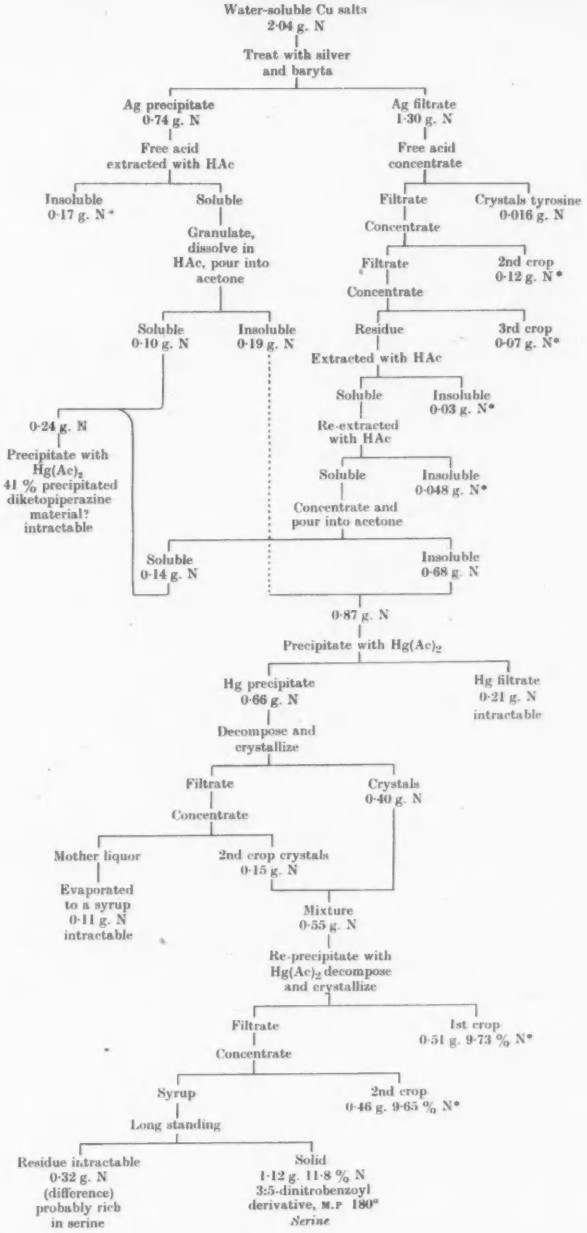
The residual syrup on long keeping became semi-solid, it was scraped out, filtered, dried for 3 weeks over conc. H_2SO_4 (wt. 1·12 g.); N, 11·8 %; moisture content 6·15 %; N in dry material 12·6 %). It was thought to be serine both from its N content and because it precipitated even in dilute solution with mercuric acetate in 50 % methyl alcohol, a property which the author has found to be characteristic of serine. It also yields a 3:5-dinitrobenzoyl derivative, M.P. 180°, mixed M.P. with a known *l*-serine derivative 181°, softens 179° (serine derivative, M.P. 183°).

The steps taken in the investigation of the water-soluble copper salts from the reprecipitation of the dicarboxylates, containing originally 2·04 g. N, are indicated schematically below (Diagram 2).

Examination of copper aspartate. This fraction which, from an examination of the feathery crystals of the copper salt, appeared to be homogeneous, was examined because the water content, 24·6 %, corresponded with 3·25 mol. of water of crystallization, whereas the figure quoted in the literature is 4·5 mol. The copper aspartate left, anhydrous wt. 7·12 g., was dissolved in 60 ml. *N* HCl, freed from copper and the solution evaporated and crystallized. By a complex process of crystallization from water and from alcohol, the free amino-acid was found to be a mixture. There was isolated 2·25 g. aspartic acid; M.P. >320°; C, 35·78; H, 5·00, N, 10·39 %: theory C, 36·08, H, 5·26, N, 10·53 %, and also 2·30 g. of an amino-acid, M.P. 194°, softening at 191°, which gave on analysis figures corresponding to glutamic acid: found, C, 40·2, H, 6·56, N, 9·76 %: theory C, 40·7, H, 6·90, N, 9·52 %: however, on treatment with 3:5-dinitrobenzoyl chloride it gave a derivative, M.P. 202°, softening at 200°, whereas natural glutamic acid yields a derivative M.P. 104°. As mentioned later in this paper this amino-acid is thought to be racemic glutamic acid, and what was thought to be copper aspartate is apparently a mixture or a double salt of copper aspartate and copper *r*-glutamate approximately in the ratio 1 : 1. This of course may be a phenomenon peculiar to this hydrolysis, but it is of great importance to ensure the purity of copper aspartate as aspartic acid is so frequently estimated as its copper salt.

Examination of the crops of glutamic acid hydrochloride. The details of the routine examination of these five crops are given in Table 1, and need no comment except to point out the low M.P. of crop 5 and to note its low rotation.

Diagram 2



All the fractions marked with an asterisk were found to give a 3:5-dinitrobenzoyl derivative M.P. 204°. This is the material which is considered to be *r*-glutamic acid. See p. 426 for full description.

Attempts were made to prepare the *p*-nitrophenylosazone of malic semi-aldehyde according to the method of Dakin [1918] from all these five crops in order to see if there was any *β*-hydroxyglutamic acid in the fractions, for its hydrochloride is known to be insoluble in HCl: 0·15 g. of each crop was used in the experiment and in each case crops 1, 2, 3 and 4, yielded no crystals from nitrobenzene. Crop 5 yielded 0·5 mg. of a dark red crystalline solid, M.P. 299° (uncorr.). This corresponds to a yield of 0·17 %, assuming crop 5 to be hydroxy-glutamic acid for calculation purposes. The substance gave only the faintest blue colour with alcoholic NaOH, whereas *p*-nitrophenylosazones are said to give an intense blue colour with this reagent. These results were not very encouraging, and the fractions were put on one side.

Later [Town, 1941], it was found that glutamic acid yielded a well-characterized 3:5-dinitrobenzoyl derivative and so this derivative was prepared from each crop (see Table 1); 0·80 g. of material was used in each case equivalent to 1 g. acid chloride; only 0·4 g. of crop 5 was used.

	Wt. of deriv. g.	M.P. °	Yield %
Crop 1	0·62	104	40
Crop 2	0·62	104	40
Crop 3	0·61	104	39·4
Crop 4	0·59	104	38·1
Crop 5	0·40	203	51·6 calc. as glutamic acid deriv.

It can be seen from the M.P. and yield that crops 1–4 are undoubtedly homogeneous and consist of glutamic acid hydrochloride. Crop 5 is, however, quite different for it yields a derivative of much higher M.P. and also in a 25 % greater yield from only half the starting material. Careful attempts, not detailed here, have been made by fractionation to see if other substances were present, but no other 3:5-dinitrobenzoyl derivative than the one with M.P. 203° was isolated from this fraction.

After this, the same derivative was prepared from all the fractions which had been obtained in the attempt to isolate hydroxyglutamic acid from the residues from the precipitation of the glutamic acid as hydrochloride. It can be seen below that most of them consisted almost completely of the amino-acid yielding the 3:5-dinitrobenzoyl derivative with M.P. 203°. A similar amino-acid was also isolated by fractional crystallization of the aspartic acid obtained from the copper aspartate.

Examination of the amino-acid yielding a 3:5-dinitrobenzoyl derivative with M.P. *203–204°*

It is first of all important to study the disposition of this acid in the scheme of separation employed.

It occurs in crop 5 of the glutamic acid separation (Table 1); therefore its hydrochloride is sparingly soluble in strong HCl. It is present in the copper aspartate fraction, and therefore it either forms an insoluble double salt with copper aspartate or else its copper salt is itself sparingly soluble. It occurs both in the silver precipitate and filtrate on treatment with silver and baryta, but it is present to a greater extent in the filtrate; precipitation is thus only partial. The amino-acid is also partially soluble in glacial acetic acid.

The weights of material obtained from the various sources (see diagram 2) and also the yields of the 3:5-dinitrobenzoyl derivative obtained from the various fractions in which it occurs are indicated below. It is interesting to note

that the yield of derivative is usually of the order 50–60 %, compared with one of 40 % in the case of *l*(+)-glutamic acid (see p. 426).

	Free amino-acid g.		Yield of deriv. %	
As hydrochloride 3·0 g. (crop 5)	2·4		51·6	
From Ag precipitate (diagram 2)	1·7		26	
From Ag filtrate—2nd crop	1·5		24	
From Ag filtrate (Table 3)—3rd crop	1·1		69	
0·79 g. insol. in glac. HAc	0·8		62	
0·51 g. from precipitation with $HgAc_2$	0·5		58	
0·46 g. with $HgAc_2$	0·46	impure	17	neglected
	8·0			

Also the copper aspartate (q.v.) yielded 2·3 g. of the same amino-acid giving a derivative melting at 202°. There have thus been isolated 10·3 g. of the material corresponding to a yield of 1·93 % of the dry protein or 1·07 % of the total N.

The hydrochloride of the amino-acid. Crop 5 of the hydrochlorides (Table 1) was dissolved in 17 ml. H_2O and saturated with HCl gas; on cooling and rubbing, it crystallized in fine needles, M.P. 194°, wt. 0·815 g. (wt. of starting material 1·570 g.). The mother liquor on evaporation to $\frac{1}{4}$ bulk was brown in colour and on cooling yielded a second fraction, wt. 0·29 g.; M.P. 194°. The two fractions were mixed and recrystallized and the derivative still had M.P. 194°. (Found: C, 32·87; H, 5·30; N, 7·53; Cl, 19·26 %. $C_5H_9O_4N.HCl$ requires C, 32·7; H, 5·45; N, 7·64; Cl, 19·35 %.) The amino-acid hydrochloride thus corresponds in composition with glutamic acid hydrochloride. A M.P. of 194° is among those quoted in the literature for glutamic acid hydrochloride, but it was not then appreciated that it could be the racemic variety.

The 3:5-dinitrobenzoyl derivative. This derivative was prepared in a similar manner to that of glutamic acid [Town, 1941]. It crystallizes in very thin hexagonal plates, M.P. 204°, which have a great tendency to overlay one another so that it is apt to crystallize in a large mass with only the ends of the crystals apparent. The crystal plates are so thin that good definition is difficult to obtain in photography. Photographs of the crystals have been obtained and copies can be sent to interested readers. The ordinary glutamic acid derivative, M.P. 104°, crystallizes in masses of very long fine needles. A 0·8 % solution in glacial acetic acid of the derivative melting at 204° gave no detectable rotation.

This derivative has no water of crystallization (the ordinary glutamic acid derivative crystallizes with 1 H_2O). (Found: C, 42·99; H, 3·15; N, 12·10 %; a substance of formula $C_{12}H_{11}N_3O_9$ has C, 42·4; H, 3·23; N, 12·30 %; which would correspond with the derivative from an acid of the formula $C_5H_9O_4N$.)

The free amino-acid. The residues of the free amino-acid, after the preparation of the 3:5-dinitrobenzoyl derivatives from all of them, were mixed (wt. 1·633 g.) and crystallized from 10 ml. H_2O; crystals readily separated on cooling, wt. 1·334 g., M.P. 203·5° softening slightly at 200·5°. The mother liquor on evaporation to 3 ml. deposited more crystals; wt. 0·042 g., M.P. 202·5°. The crystals were tetrahedral in shape, very hard to the touch, and practically insoluble in cold water. (Found: C, 41·02; H, 6·06; N, 9·38 %. $C_5H_9O_4N$ requires C, 40·7; H, 6·13; N, 9·52 %.)

The free acid thus also corresponds in composition with glutamic acid.

It was also determined that the ratio of amino-N/total N was 1/1.

Racemization of known glutamic acid. It was suggested that the material might be *dl*-glutamic acid and so 15 g. pure *l*(+)-glutamic acid were racemized

by boiling in *N* NaOH and the rotation determined at 4 hr. intervals. Racemization was complete after 130 hr. boiling. The solution was decolorized with animal charcoal and after evaporation was extracted three times with 80 % alcohol. This extract in aqueous solution was saturated with HCl gas, and there were obtained 4·2 g. of pure *dl*-glutamic acid hydrochloride, M.P. 213° (sharp).

This yielded a 3:5-dinitrobenzoyl derivative (49 % yield), M.P. 104°—this seems to be the point at which the material dissolves in its own water of crystallization. The anhydrous derivative has M.P. 182°, softening at 179°. (Found: 5·05, 5·10 % H_2O corresponding to 1 H_2O per mol., similar to the glutamic acid derivative.) Thus the amino-acid isolated is not *dl*-glutamic acid.

The 3:5-dinitrobenzoyl derivative was also prepared from a sample of *d*(−)-glutamic acid kindly supplied by Prof. A. C. Chibnall; 0·1 g. of the acid gave a 62 % yield of the derivative which had M.P. 105°, mixed M.P. with known 104° derivative, 104°, softened at 101°. Loss in weight at 110° 5·12 %.

The possibility also existed that the amino-acid isolated might be methionine, which with a formula $C_5H_{11}SO_2N$ would give very similar analytical figures, but tests for S by Na fusion were negative. Before it was considered that the acid might be *r*-glutamic acid, it was thought probable that it might be either methylaspartic acid or the lactone of hydroxyglutamic acid and a decision could only be made by oxidation experiments.

Oxidation of the 3:5-dinitrobenzoyl derivative. It was decided to use this derivative in order to protect the amino group. 169 mg. of the derivative were dissolved in 5 ml. glacial acetic acid and the boiling solution titrated with 33·2 ml. *N*/10 $KMnO_4$ over the space of 5 min., equivalent to 2 atoms oxygen per mol. of derivative. The MnO_2 was filtered off and the solution evaporated on the water bath; solid separated when the volume was 3 ml.; wt. 47·5 mg., M.P. 164°, softened 159°. This, on crystallizing from 5 ml. boiling water, gave 21·4 mg. derivative, M.P. 182·5°, softening at 182°, crystallizing in rosettes of fine needles. (Found: C, 40·95; H, 2·57; N, 12·43%. $C_{11}H_9N_3O_9$ or $C_4H_6O_4N.C_7H_3N_2O_5$ would require C, 40·45; H, 2·75; N, 12·43 %.) This derivative was not natural aspartic acid 3:5-dinitrobenzoyl derivative, however, in spite of its similar M.P., because a mixed M.P. with the derivative (M.P. 184·4°) gave M.P. 110° softening at 90°. Work is continuing on its identity.

Titration of the acid with alcoholic NaOH. 60 mg. of the acid in 5 ml. H_2O and 50 ml. ethyl alcohol were titrated with *N*/10 NaOH using thymolphthalein as indicator. As a check 60 mg. ordinary glutamic acid were treated similarly. Both solutions were back-titrated with *N*/10 HCl using methyl red as indicator. In both cases the ratio of $COOH/NH_2$ groups was 1·88/1 indicating that the acid is a dibasic monoamino-acid. The mol. wt. of the acid is 147·8 (glutamic acid 147).

The silver salt. The solution from the above titration was treated with an exact equivalent of *N*/10 $AgNO_3$ to free from Cl^- (wt. AgCl 14·5 mg.; should be 67·5 mg. thus some AgCl is retained in solution). The solution was decolorized by boiling with 0·25 g. charcoal and evaporated to a syrup; 150 mg. $AgNO_3$ in 2 ml. H_2O were added and the yellowish-white silver salt filtered off; wt. 120·2 mg. (theory is 147 mg. silver salt). This silver salt went black within an hour or so, and contained 60·1 % Ag. It was dissolved (112·3 mg.) in 5 ml. *N*/10 HNO_3, filtered from a trace of black residue (3·5 mg.) and brought to *p*H 7–8 (bromothymol blue) by adding 4 ml. *N*/10 NaOH; a whitish precipitate formed (46·7 mg.) which contained 8·49 % H_2O and 58·5 % Ag in the anhydrous salt. This salt also blackened readily on exposure to light. An acid of mol. wt. 147 would give a salt with 59·8 % Ag.

The copper salt. 28·6 mg. of acid in 5 ml. H_2O were boiled with 24 mg. $Cu(OH)_2$ (theory 19·3 mg.) and filtered; the deep blue solution on evaporation gave a blue glass, readily soluble in water, and readily precipitated by adding an equal volume of methyl alcohol as a bulky light blue amorphous powder. % Cu, 19·7. Anhydrous copper glutamate contains 30·3 % Cu, if Cu/N is 1/1.

Electrometric titration. The author's thanks are due to Prof. C. R. Harington for kindly arranging for an electrometric titration to be carried out on the amino-acid, and assuming the mol. wt. to be 147, the following pK values were determined. The figures for glutamic and aspartic acids are given for comparison.

	Amino-acid	Glutamic acid	Aspartic acid
pK_1	2·25	2·19	2·09
pK_2	4·17	4·28	3·87
pK_3	9·44	9·66	9·82

The figures afford strong evidence that the acid isolated is identical with glutamic acid.

Reduction. Attempts were made at reduction of the amino-acid using hydrogen in the presence of Pd adsorbed on charcoal as a catalyst to eliminate all possibility of it being the lactone of hydroxyglutamic acid. No evidence of the absorption of hydrogen was observed.

Oxidation. As the amino-acid thus undoubtedly appears to be a glutamic acid, it was decided to oxidise it with chloramine-T or other source of hypochlorous acid to β-cyanopropionic acid and thence by hydrolysis to succinic acid. Free hypochlorous acid was prepared by distilling a solution of Cl_2 water after treating it with mercuric oxide. On the basis that 1 g.-mol. of glutamic acid requires 2 mol. of HOCl (i.e. 4 equiv.) for oxidation to cyanopropionic acid, the exact equivalent of HOCl was added. 14·7 mg. of acid in 10 ml. solution were treated with 1·31 ml. HOCl (0·307 N) at 40°. After 30 min. the solution still gave a blue colour with starch-iodide paper, which became negative after warming for 2 min. The solution was evaporated on a water bath; 5 ml. 5N HCl were added and the solution evaporated to dryness. On keeping crystals formed; these were extracted with ether and yielded 5·7 mg. succinic acid, M.P. 181°, mixed M.P. with authentic succinic acid, 182–184°. A control experiment with 14·7 mg. of $l(+)$-glutamic acid yielded 6 mg. of succinic acid, M.P. 182–184°.

p*H of aqueous solution of the acid.* The pH of $M/100$ solutions (14·7 mg. in 10 ml. solution) of known and new glutamic acids were determined electrometrically and found to be respectively 3·35 and 3·31. This also tends to confirm the suggestion that the unknown acid is a glutamic acid.

Hydrolysis of 3:5-dinitrobenzoyl derivative of the acid. 210 mg. of the derivative were heated in a sealed tube at 120° with 5 ml. conc. HCl for 1 hr.; after cooling, the 3:5-dinitrobenzoic acid was filtered off: wt. 92 mg., 70 % theory. The solution was evaporated to give the free amino-acid hydrochloride (wt. 133 mg.), and this was dissolved in 9 % HCl and made up to 3 ml. The rotation was determined and the concentration of amino-acid by Kjeldahl analysis. Concentration, 95·6 mg. per 3 ml. solution; rotation, −0·15°; hence $[\alpha]_D = -2·36°$. Some doubt is felt about this rotation as the solution was slightly turbid and in the 2 dm. tube used it was very difficult to read; however, the value −0·15° is the mean of 24 readings. The value may well have been zero when compared with the value obtained below with known glutamic acid.

A similar experiment was carried out on the 3:5-dinitrobenzoyl derivative of known $l(+)$-glutamic acid. 220 mg. hydrolysed yielded 114 mg. 3:5-dinitrobenzoic acid (78 % theory) and the amino-acid was treated as above. Concen-

tration of solution 76·7 mg. per 3 ml.; rotation +0·64°; and $[\alpha]_D = +12·5°$. If this specific rotation is accurate and this solution was also slightly turbid, then it would appear that in the benzoylation process the *l*(+)-glutamic acid was racemized to the extent of 60 %; i.e. 30 % of the material was converted into its opposite enantiomorph, which however existed as the *dl* mixture and not as the racemic compound, for the 3:5-dinitrobenzoyl derivative had M.P. 104°, whereas the new amino-acid isolated yielded a derivative M.P. 204° and this is thought to be the racemic compound.

Re-benzoylation of the amino-acids obtained above. After the rotations of the free amino-acid hydrochlorides had been determined, the HCl was removed by evaporation and the residues treated with 0·15 g. of 3:5-dinitrobenzoyl chloride and NaOH in the usual manner.

The amino-acid from the derivative which had M.P. 204° gave a product which, after benzene extraction and crystallization of the benzene-insoluble portion from water, yielded 50 mg. of the 3:5-dinitrobenzoyl derivative and this still had M.P. 204°. The yield was 42·6 % calculated on the 85 mg. of acid which were left for this purpose. This corresponds with yields of the order of 50 % which were obtained using 10 times the quantity of starting material and would appear to indicate that no change has been caused by the repeated benzoylation.

The amino-acid from the derivative with M.P. 104°, which is that obtained from *l*(+)-, *d*(−)- and *dl*-glutamic acids, yielded by this same treatment 8 mg. of M.P. 204° derivative (a yield of 4·5 %); a mixed M.P. with known 204° derivative gave M.P. 201°, shrinking at 198°. Nothing more could be crystallized from the mother liquor, although careful attempts were made to increase the yield.

This experiment confirms the fact that the amino-acid which yields a 3:5-dinitrobenzoyl derivative M.P. 204° is a glutamic acid and also that it is the racemic compound, for all the other possibilities have been tried and found to yield the 104° M.P. derivative.

Although the *l*(+)-glutamic acid does, after two benzoylations, yield small quantities of the derivative with M.P. 204°, it is important to note the small yield in this case, only 4·5 %, compared with a yield of 42·6 % in the case of the amino-acid which originally gave the derivative with M.P. 204°. It can thus be seen that slight conversion of ordinary glutamic acid into the racemic compound does take place after repeated benzoylation, but compared with the yields obtained in the course of this analysis (from 50 to 60 %) after only *one* benzoylation it is negligible.

All the known experimental facts can be reconciled if it is presumed that the original amino-acid giving a 3:5-dinitrobenzoyl derivative M.P. 204° was racemic glutamic acid and owing to the great difficulty experienced in inducing ordinary *l*(+)-glutamic acid and also known *dl*-glutamic acid to form such a derivative, it is suggested that the *r*-glutamic acid is a hydrolysis product and not an artefact. It is hoped, when opportunity offers, to carry out investigations to determine the equilibrium conditions between *dl*- and *r*-glutamic acids.

Conclusion

The dicarboxylate fraction of gliadin has been exhaustively examined and the partition of the nitrogen in the various fractions is shown in the following balance sheet. Corrections have not been made for the nitrogen used in Kjeldahl determinations, AM the nitrogen otherwise unaccounted for is considerably greater.

Nitrogen distribution in the dicarboxylate fraction of gliadin

			Original N content
Isolated			
l(+)-glutamic acid	19·0 g.		24·6 g.
dl(mixture)-glutamic	0·35 g.		
r(compound)-glutamic	0·97 g.		
		20·32 g.	
Non-dicarboxylate N after reprecipitation of mother liquors with baryta and alcohol		1·46 g.	
Ammonia present		0·55 g.	
Aspartic acid (2·25 g.)		0·24 g.	
Serine		0·13 g.	
Tyrosine		0·01 g.	
		22·71 g.	
Losses			
On AgCl	0·01 g.		
On $BaSO_4$ (2nd precipitation)	0·26 g.		
On excess $Cu(OH)_2$	0·33 g.		
On CuS	0·03 g.		
On Ag_2S	0·02 g.		
Soluble in acetone	0·24 g.		
$Hg(Ac)_2$ filtrate	0·08 g.		
Loss in crystallization of aspartic acid	0·04 g.		
		1·01 g.	23·7(2)
Unaccounted for		0·89 g.	
		24·6(1) g.	

Thus 23·7 g. N out of a total of 24·6 g. are accounted for, or 96·4 %, although just over 4 % represents unavoidable losses on precipitates. The only amino-acid present besides glutamic and aspartic acids, except for 1·46 g. N which did not precipitate with baryta and alcohol on the second treatment with this reagent (and which was mixed with the main bulk of copper salts), is a small quantity of serine. This is a very soluble amino-acid and its presence no doubt had a very profound effect on the crystallization of the final fractions. The presence of serine always tends to prevent the crystallization of other amino-acids.

Summary

An investigation into the dicarboxylate fraction of gliadin, obtained by precipitating the dicarboxylates from the hydrolysed protein by means of baryta and alcohol, has been completed.

The yield of glutamic acid amounts to 40·4(3) % of the dry protein, or to 20·7 % of the total N.

Besides the ordinary *l*(+)-glutamic acid, there was isolated a new variety of glutamic acid which is believed to be racemic glutamic acid. This is thought to be a definite hydrolysis product and not an artefact. The ratio of racemic to *l*(+)-glutamic acid is 1/20.

The aspartic acid was isolated as a well-crystallized copper salt, but this was found to be a mixture, and to approximate to a double salt of copper *r*-glutamate and copper aspartate. This result may be peculiar to this analysis, but it indicates the necessity of ensuring the purity of copper aspartate in this method of estimation.

The yield of pure aspartic acid was 0·43 % of the dry protein, or 0·26 % of the total N.

The only other amino-acid present was serine, which was isolated to the extent of 0·18 % of the dry protein, or 0·14 % of the total N. It was identified

by its ready precipitation with mercuric acetate in 50 % methyl alcohol, and by its 3:5-dinitrobenzoyl derivative. This amino-acid no doubt accounted for the intractability of the mother liquors from the glutamic acid.

There seems no ground for the belief that baryta and alcohol, at least after a second precipitation with this reagent, bring down considerable quantities of other amino-acids.

Although only just published, a large part of this work was carried out during the tenure of an Alexander Brown Coxe Research Fellowship at Yale University, Connecticut, U.S.A., during the year 1931–32. Publication was delayed owing to the number of fractions whose identity had not been established. The work was continued at St Bartholomew's Hospital Medical College.

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43. AN ANALYSIS OF BENCE-JONES PROTEIN

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Bence-Jones protein, which is characterized by precipitation from solution at low temperatures (45–50°) and resolution of the coagulum on boiling, is excreted in the urine in about 70 % of the cases of multiple myelomatosis and occasionally also in allied diseases of the bone marrow and blood-forming organs. Of some seven hundred papers on myelomatosis which have appeared since Bence-Jones [1848] first noted the excretion of a peculiar substance in a case of 'mollities ossium', relatively few have dealt in detail with the chemistry of the protein. The purpose of the present communication is to give the results of an analysis of the amino-acid content of a sample of Bence-Jones protein obtained from a case of multiple myelomatosis which recently came under observation in the terminal stages, in the Tropical Ward of the Royal Infirmary, Liverpool; other features of the case will be discussed elsewhere.

The urine, which was collected daily under toluene for about 3 weeks immediately before the death of the patient, showed the typical reactions of Bence-Jones protein and was free from normal plasma proteins. After storage at 0° the different samples were filtered and mixed; part was reserved for isolation of the protein in soluble form, and the rest was coagulated for analysis.

Experimental

As the amount of material for amino-acid analysis was small, attention was confined to colorimetric and small scale isolation methods. No attempt was made to apply the general solubility method devised by Bergmann & Stein [1939]. All the colorimetric analyses were carried out by means of a Hilger 'Spekker' Absorptiometer. In using this instrument the drum reading obtained by comparing the coloured solution with the appropriate blank is referred to a calibration curve similarly prepared from standard solutions of the pure constituent being estimated. The light filter is so chosen as to give maximum sensitivity. Solutions for the 1 cm. cups should be made up to 10 ml. All the reagents used were of A.R. quality or were purified by standard procedures and were freshly made up in solution. Blank tests were carried out where necessary and all the procedures were submitted to preliminary test against standard protein preparations. Unless otherwise stated, the results obtained with Bence-Jones protein are the mean of two or more determinations and refer to the moisture- and ash-free material.

Preparation of Bence-Jones protein. The *p*H of the urine was adjusted with acetic acid to the point of maximum precipitation in the cold and the coagulation completed by heating to 60° for 30 min. Exhaustive washing of the protein by centrifuging was carried out by allowing it to stand 1–2 days in the wash liquor and repeating this several times afresh—first with very dilute acetic acid, then

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water, water at 60°, aqueous alcohol, absolute alcohol, and finally light petroleum twice overnight.[1] The latter was repeated by extracting twice for a day in a Soxhlet apparatus and the protein was then air-dried under cover and finally in a desiccator over H_2SO_4.

Methods

Moisture. A sample was dried to constant weight at 108° (about 4 hr.) in a silica crucible.

Ash. The residue from the moisture determination was ashed over a Bunsen flame and then kept at 600° for 2 hr. in a furnace.

Total N. By micro-Kjeldahl analysis. In a repeat experiment, with fresh standard solutions and $CuSO_4$-K_2SO_4 mixture as catalyst, the results were identical with those originally obtained by H_2SO_4-H_3PO_4-$K_2S_2O_8$ digestion.

Amide-N. About 0·07 g. was hydrolysed in a 6 × 1 in. test tube by heating for 6 hr. with 5 ml. 2*N* HCl [cf. Lugg, 1938, 3]. Part of the HCl was removed by concentration *in vacuo* and after neutralizing to phenolphthalein and adding 0·15 g. K_2CO_3 the solution was rapidly aerated for 2 hr. at 30° [Denis, 1910] and the NH_3 determined in *N*/50 acid in the usual manner.

Humin-N. The hydrolysate prepared for the determination of the basic amino-acids was filtered through a Whatman No. 1 paper and the residue washed well with distilled water and analysed by the Kjeldahl procedure.

Total S. Wet ashing by the Benedict-Denis method is known to yield faulty results as compared with the standard Parr bomb method [Painter & Franke, 1936; Masters, 1939] or the peroxide fusion method [Bailey, 1937] of which use was made in the present case following the directions of Lugg [1938, 2]. The usual procedure for the removal of Group 3 metals was carried out before precipitating $BaSO_4$.

Total P. About 0·05 g. protein was digested with 1 ml. conc. H_2SO_4 with the dropwise addition of a little perhydrol (Merck). The digest was neutralized and brought to 7 ml. and analysed by the method of Berenblum & Chain [1938]. Filter No. 3 (orange).

Tyrosine and tryptophan

The colorimetric method of Folin & Marenzi [1929] was used as adapted from that of Folin & Ciocalteu [1927]. From the limited data available, this appears to give substantially the same results as the later modification suggested by Lugg [1937]. In three determinations with about 0·08 g. protein, the time of hydrolysis and other details were varied. Separation of the tryptophan as the mercury complex was allowed to proceed overnight at 0° in each case. The tyrosine solutions were read immediately on mixing with the reagents as turbidity developed within a few minutes. The 30 min. development of the tryptophan colour with the Folin reagent was carried out in a bath at 20° or at room temperature and saturated solutions of Na_2CO_3 made at 18° and 25° respectively were used; the corresponding calibration curves were identical. Filters: Tyrosine No. 7 (dark blue), tryptophan No. 1 (red).

Dr R. A. Morton and Mr T. W. Goodwin kindly carried out an independent analysis by a slight modification of Holiday's [1936] spectrophotometric method. They conclude from experiments with casein (unpublished) that in order to make the difference between the observed absorption in the ultraviolet region

[1] For determination of the S-distribution according to Baernstein [1936, 1] it is necessary to eliminate traces of alcohol and ether and avoid drying above 100°. Lugg [1938, 2] states, however, that oven-drying does not affect the results appreciably.

and that calculated from the best available chemical data fit in with the extrapolated curve showing general absorption beyond 310 mμ, it is necessary to carry out a hydrolysis of the protein with Lugg's [1938, 1] alkaline stannite reagent instead of merely dissolving it in NaOH. Apart from this modification, a preliminary determination on Bence-Jones protein in NaOH showed that the interference due to pigment was so great as to make the corrected (Holiday) result valueless; only a trace of tryptophan was evident. The more reliable results obtained on the colourless Lugg hydrolysate were: tyrosine 8·5 %, tryptophan 2·3 %. The corresponding figures obtained colorimetrically were 8·8 % and 1·4 % respectively, so that while the results for tyrosine compare well, those for tryptophan show a wide and unexplained divergence.

Sulphur distribution

The discussions by Lugg [1932; 1933; 1938, 2] and Bailey [1937] of the various procedures for differentiating the total S fraction of proteins and the difficulties attendant on the colorimetric estimation of cystine led to the adoption in the present instance of Baernstein's [1936, 1, 2] elegant and comprehensive method. By hydrolysis with conc. HI a study of the S-distribution can be completed on 0·5 g. protein or less, including the determination of methionine as well as of cystine by titration and the estimation of any volatile S (H_2S+SO_2) arising during hydrolysis from SO_4 groups, etc., by absorption in $CdCl_2$-$BaCl_2$ mixture. On this basis it is claimed that almost all the S of a number of proteins can be quantitatively accounted for. The scheme does not permit of discrimination between cystine and cysteine. Certain refinements in the method suggested by Kassell & Brand [1938] do not appear to be essential [Macara & Plimmer, 1940].

Two traps instead of one were used for the volatile S, so that the efficiency of absorption could be gauged. These consisted of 10 ml. tapering centrifuge tubes with ground glass connexions, each containing 3·5 ml. wash liquor. A trial of the procedure with casein gave satisfactory results; the volatile S (0·016 mg.) was only enough to produce a faint turbidity with $CaCl_2$-$BaCl_2$. Recoveries of the amino-acids from a known mixture gave 98 % for cystine and 90 % for methionine, in agreement with the values reported by Kassell & Brand [1938] and Lugg [1938, 2].

Application of the method to Bence-Jones protein immediately made it obvious that a relatively large proportion of the S was being volatilized. A heavy precipitate formed in the first trap as soon as boiling commenced and necessitated, at the end of the reaction, careful washing out of the inlet tube with iodate mixture to decompose the deposit of sulphide. One determination on 0·369 g. protein yielded the following results, including the correction for recovery: cystine (cysteine + cystine) 2·91 %, methionine 0·58 %, volatile S 1·56 mg. After correction of the latter value by subtracting 1 % of the methionine-S and 2 % of the cystine-S [Kassell & Brand, 1938] the figures account almost quantitatively for the total S content of the protein: (cystine + methionine)-S 69·7 %; volatile S 28·2 %; total S recovery 97·9 %.

A. By isolation *The basic amino-acids*

Arginine and lysine. The small scale adaptation by Block [1934] of the classical Kossel & Kutscher [1900] method for the determination of the basic amino-acids has been further modified recently [Block, 1938; Tristram, 1939].

The procedure advocated by Tristram for the isolation of arginine and lysine has been followed in the present investigation, since it avoids the use of excess $AgNO_3$ (which is liable to interfere with the estimation of lysine), gives greater delicacy in the adjustment of *p*H values, and incorporates the finding of Ayre [1938] that ammonium phosphotungstate is not decomposed by dilute H_2SO_4 in the presence of amyl alcohol-ether. The removal of NH_3 by NaOH before the isolation of lysine as picrate is thereby eliminated.

All the standard operations of concentrations *in vacuo*, etc., were adhered to. In the final stages of both analyses the operations were determined as usual by the total N content (Kjeldahl) of the solutions.

Histidine. While the figures recorded by Tristram lend no support to Block's [1938] contention that no more than about 75 % of the histidine present is recoverable as diflavianate, this amino-acid is not easy to deal with on account of the relatively small quantities present in most proteins and the difficulty of isolating the derivative in a state of purity. For this reason Block [1937] has suggested the use of nitranilic acid to precipitate histidine. In a later paper [Block, 1940], the recovery of histidine both from pure solutions and from protein hydrolysates is stated to be quantitative, using the factor 0·403 for the conversion of the nitranilate to histidine. This was checked on histidine solutions as follows: about 0·14 g. histidine hydrochloride monohydrate dissolved in 15 ml. water was precipitated by the addition of 0·24 g. nitranilic acid in 15 ml. methyl alcohol. The derivative crystallized immediately. The solution was left overnight in the ice-chest, filtered on a sintered glass crucible and the residue was washed with methyl alcohol and ether and dried in the air and for a short time at 108°. Recoveries were 100 % from B.D.H. histidine and 98·2 % from a slightly impure sample prepared from ox blood.

In applying this method to Bence-Jones protein, the histidine-Ag fraction was put through the whole procedure in use for the precipitation of histidine as diflavianate, i.e. removal of cystine and other impurities by successive treatment with $HgSO_4$ and $CuCO_3$. This is preferable to the shortened method proposed by Block [1938] in which the solution, after removal of Ag by H_2S, is merely treated with charcoal before addition of nitranilic acid. From the final solution (50 ml.) four 1 ml. aliquots were removed for colorimetric estimation as described below. The remainder, which was thereby calculated to contain about 19 mg. histidine, was concentrated *in vacuo* to 3 ml. in a 50 ml. distilling flask and 50 mg. nitranilic acid, dissolved in 7 ml. methyl alcohol, were added at room temperature. Crystallization was completed overnight at 0° and the precipitate collected quantitatively, as described before.

B. By colorimetric methods.

Where the available material allows only one gravimetric determination of the basic amino-acids to be made, the relative purity of the final histidine and arginine fractions provides a useful opportunity for checking the results colorimetrically, and safeguarding against the possibility of subsequent loss of material.

Histidine. Methods based on diazotization [e.g. Koessler & Hanke, 1919] have found little application. The Knoop [1908] reaction, which has been utilized by Kapeller-Adler [1933] for the quantitative estimation of histidine in pregnancy urines, has been applied recently to protein hydrolysates. An examination of recent papers on this reaction by Armstrong & Walker [1932], Földes [1936], Conrad & Berg [1937], Woolley & Peterson [1937] and Racker [1940] indicates that the procedure described by Block [1938] incorporates the most satisfactory

modifications for use in protein analysis. The solution is treated at room temperature for 10 min. with the bromine reagent, slight excess of which is then destroyed by addition of a drop of arsenite solution. 2 ml. of ammonia-ammonium carbonate solution are added and the mixture is heated in the boiling water bath for 5 min., and then kept at room temperature for a further 10 min. for the complete development of the colour. It soon became evident on analysis of the histidine-Ag fraction from edestin that reasonably consistent results could not be obtained. Unfortunately, there is no record of individual variations apart from that which is implied in Block's directions to develop both unknown and standard solutions in sets of six, and then to mix each set before comparing. Woolley & Peterson [1937] claim a 97 % recovery of histidine from a yeast hydrolysate, whereas Racker [1940], in controlled tests on urine, states that the experimental error is about 10 %.

In experiments on pure histidine solutions no improvement was effected by carrying out the second stage of the reaction, which is probably the source of the trouble, at 90–95° with the object of minimizing the spitting and uneven heating of the liquid consequent on the rapid evolution of NH_3. An apparent improvement in this respect was achieved by the use of thin-walled, wider bore tubes ($6 \times \frac{3}{4}$ in.). In studying the effects of temperature and heating period on the intensity and stability of the colour, it was clearly not advisable to aim for conditions of maximum intensity, since this was still not attained after 22 min. heating at 65°, 75° or 100°. The results suggested that the original directions to heat in the boiling water bath for 5 min. are preferable in that the colour is stable or almost so for about 10 min. after taking the first reading, though not for an hour as stated by Kapeller-Adler [1933]. For longer periods at 100° the colour is more intense but fades with increasing rapidity. Colours which were stable for at least 20 min. were also attained by heating at 75° for 12–17 min., but such conditions are less suitable for routine analysis. Under all the other conditions tested the solutions were unstable.

In following Block's directions (5 min. heating in a boiling water bath, etc.) for the construction of a calibration curve with the minor modifications suggested by the above, the mean deviation in the absorptiometer readings (Filter Nos. 5+6) averaged over each set of five determinations was 3·3 %. Over the range investigated (0·4–1·4 mg. histidine) the mean values for each set fell exactly on a straight line. Treatment of the histidine-Ag fraction of Bence-Jones protein with charcoal [Block, 1938] after removal of Ag with H_2S was unsatisfactory. The clear colourless solution gave a white precipitate on adding bromine and finally a turbid brown-violet solution. After further purification of the mother liquor in the usual manner with $HgSO_4$ and $CuCO_3$ the colour developed satisfactorily. The spread of the results was much greater than before, however, the four absorptiometer readings being 0·294, 0·271, 0·278, 0·257.

Arginine. Further comments on the procedures of Weber [1930] and of Jorpes & Thorén [1932] for the colorimetric estimation of arginine by means of the Sakaguchi reaction have recently been made by Fisher & Wilhelmi [1937], Davenport *et al.* [1938] and Thomas *et al.* [1939], and the choice of certain empirical factors has been more carefully defined. The time assigned to the first stage of the reaction, between arginine and the alkaline α-naphthol, before the addition of hypobromite, varies from 2–3 min. [Weber, 1930] to 1 hr. [Jorpes & Thorén, 1932]. A constant 0·20 ml. standard hypobromite solution (followed after a certain interval by 1 ml. 40 % urea to destroy the excess) was used by Jorpes & Thorén [1932] in developing the colour, and has been found satisfactory by Fisher & Wilhelmi [1937] and Davenport *et al.* [1938]. Thomas *et al.* [1939],

however, have utilized Weber's [1930] suggestion that the optimum hypobromite concentration should be found by trial; this modification, which is designed for use with the Sheard-Sanford Photelometer, eliminates the preliminary separation of arginine from certain other amino-acids, etc., which interfere with the colour development, and is stated to be directly applicable to protein hydrolysates. For each standard and unknown, the amount of hypobromite is varied until the light absorption remains constant for about 10 min., i.e. the fading of the red colour, which is stable for not more than 2 min., is exactly compensated by the increasing yellow decomposition colour, so that on extrapolating to zero time the reading may be taken to represent 100 % red colour. The safest and most convenient interval allowable between the addition of hypobromite and that of urea has been fixed by Davenport *et al.* [1938], in the light of their experience with the similar estimation of glycocyamine, as 15 sec., in conformity with the earlier recommendations of Jorpes & Thorén [1932]. This allows of more accurate working than the shorter period of 4 sec. used by Weber [1930].

Following the directions of Thomas *et al.* [1939], the amount of hypobromite required to give a satisfactory colour with an aliquot containing 0·12 mg. arginine from the arginine fraction of edestin was found to be 0·20 ml. This amount was optimal also for standard solutions containing 0·08–0·20 mg. arginine. It was difficult to determine with consistency the small range over which the hypobromite could safely be varied so as to give readings which remained constant. On the other hand, excellent agreement was obtained in duplicate determinations, provided that the solutions were read immediately after making up to volume. This point was brought out in the actual determination on the appropriate fraction from Bence-Jones protein. Four groups containing 10 equal aliquots were treated with 0·20, 0·25, 0·30 and 0·35 ml. hypobromite respectively. All the readings were constant within the limits of the experimental error, viz., 1 %.

The method finally adopted, using the solutions recommended by Thomas *et al.* [1939], is as follows. The reagent bottles which are kept in the ice-chest are placed in a bath at 0°. A suitable aliquot of the arginine-Ag fraction (not more than 5 ml.) containing about 0·05–0·20 mg. arginine is placed in a thin-walled $6 \times \frac{3}{4}$ in. test tube graduated at 10 ml., made up to 5 ml. with distilled water and cooled in a separate ice-bath for 30 min. 1 ml. NaOH and 1 ml. α-naphthol are added and the tube replaced in the bath for 5 min. with occasional shaking. 0·20 ml. standard hypobromite solution is added and after exactly 15 sec. vigorous shaking 1 ml. 40 % urea is added with further shaking. The solution is brought to 10 ml. with water and mixed. After warming the tube slightly in the hand to prevent subsequent condensation of moisture, the solution is transferred to the absorptiometer cell as quickly as possible so that the diaphragm can be adjusted within 60–70 sec. after the addition of hypobromite. In adding the reagents the hypobromite is sucked up several times to cool the pipette (straight 1 ml. graduated to the tip) and is then blown directly into the liquid; after this has been shaken the pipette is 'blown off' against the inside of the tube just above the liquid. This allows sufficient time for further mixing before adding the urea solution similarly, blowing this out once from an Ostwald pipette. Holders for the pipettes were not found necessary.

Filter combination Nos. 5+6 is the most sensitive.

Summary of analysis of the basic amino-acids in 2·490 g. Bence-Jones protein

With appropriate corrections for aliquots removed for N determination and colorimetric analysis and for the solubility of arginine flavianate and lysine picrate and the incidental losses of procedure [Tristram, 1939], the results are as follows:

		Wt. found (mg.)	Mean % of protein
Histidine:	By nitranilate	21·3	0·90
	Colorimetrically	23·6	
Arginine:	By flavianate	89·5	3·64
	Colorimetrically	91·5	
Lysine*:	By picrate	101·0	4·04

* The lysine picrate exploded at 256° and after recrystallization at 266°.

It may be noted that the minimum mol. wt. of the protein calculated from the histidine content (0·9 %) is 17,200; or assuming 2 mol. histidine per mol. protein, 34,400. This agrees well with the value of 35,000 determined by Svedberg & Sjögren [1929].

Phenylalanine

The colorimetric nitration method of Kapeller-Adler [1932] appears to be the only small scale method which has been proposed for the determination of phenylalanine. Jervis *et al.* [1940] have suggested, contrary to the original procedure described by Block [1938], that the removal of histidine and tyrosine may be omitted if the final solution is examined with a suitable filter. The full procedure is outlined here as conducted with slight modifications on one-fifth of the weight of protein suggested by Kapeller-Adler.

0·4 g. protein is refluxed for 20 hr. with 5 ml. 25 % H_2SO_4 in a micro-Kjeldahl flask. The humin is removed by centrifuging and washed 3 times with cold water. The solution and washings are brought to 40 ml. (filtered if necessary) and three 10 ml. aliquots are placed in 15 ml. centrifuge tubes and treated with 0·5 ml., 1·0 ml. and 1·5 ml. respectively of 10 % aqueous phosphotungstic acid. Each mixture is well stirred and the precipitate partially dissolved by warming the tube in a water bath. The solution is allowed to cool overnight, the precipitate washed with very dilute H_2SO_4 and the filtrate and washings in each case filtered through a small paper into a 50 ml. beaker, the outside of which is marked at the 10 and 20 ml. levels. The filtration ensures the elimination of phosphotungstate scum which is not easily centrifuged down. The solution is treated in the cold with $N/10$ $KMnO_4$ in small portions till the mixture is faintly coloured and then concentrated over a small flame. This operation is too tedious on the water bath. From time to time $KMnO_4$ solution is added to ensure that excess is present. When a volume of about 4 ml. is reached, further concentration to the oily stage (1–2 ml.) is effected very cautiously. 2 ml. of 10 % KNO_3 in conc. H_2SO_4 are added to the cooled mixture, the whole heated for 20 min. on the water bath and finally brought to about 6 ml. The beaker is next cooled in an ice bath and 5 ml. 15 % hydroxylamine hydrochloride added dropwise with mixing. After cooling to 0° again conc. NH_4OH is added slowly with mixing to a volume of about 20 ml. and the solution is transferred to a 25 ml. flask and brought to volume with conc. NH_4OH. The contents are thoroughly mixed and the development of the colour completed by placing the flask in a bath at 40° for 5 min. and then in an ice bath for a further 15 min. The three solutions thus prepared are read with the No. 6 filter (light blue). The determination is repeated

if necessary using only the optimum amount of phosphotungstic acid. Occasionally a solution may develop a brownish shade and should be discarded. A slight deviation in the shade of the unknown from that of the standard solution which would make matching difficult in the visual type of colorimeter can be ignored. It was not found necessary to submit the protein hydrolysate to preliminary fractionation, as recently suggested by Macara & Plimmer [1940] who were unable to develop satisfactory colours with dephosphocaseose.

For the 1·cm. cell a suitable range of values for the calibration curve is 1–8 mg. phenylalanine.

Hydroxyproline

No small scale gravimetric method has yet been developed for the determination of hydroxyproline. The colorimetric method of Waldschmidt-Leitz & Akabori [1934] for the micro-estimation of both proline and hydroxyproline in the same sample, which is developed from that of Lang [1933], requires steam distillation in a specially designed apparatus and yields a value of only 8·9–9·4 % for the hydroxyproline content of gelatin as compared with the accepted value of 14·4 % [Bergmann, 1935]. The principle of oxidizing the hydroxyproline and coupling the product with isatin has been modified along simpler lines by McFarlane & Guest [1939] who use as the oxidizing agent alkaline hydrogen peroxide in the presence of a small amount of $CuSO_4$ as catalyst. This method is stated to yield a value of 14·65 %.

Using this procedure on a sample of grade B gelatin (*a*) 0·5 g. refluxed 25 hr. with 8 ml. 20 % HCl, (*b*) 0·5 g. autoclaved with 10 ml. 3*N* HCl for 6 hr. at 145°, lower but identical values in each case of 10·4 % were obtained. As the hydroxyproline contents of most proteins are much less than that of gelatin, the colours developed from more concentrated hydrolysates (with appropriate dilution after development) were compared with those prepared according to the original procedure, but were in each case found to be less intense; no improvement was effected by increasing the concentration of reagents. Hence, where possible the final dilution should be avoided, the colour being developed in a larger volume of solution to enable the 4 cm. cells to be employed in the absorptiometer (Filter No. 6).

Tests made on Bence-Jones protein ((*a*) 0·25 g. refluxed 24 hr. with 5 ml. 20 % HCl and the hydrolysate brought to 25 ml., (*b*) 0·1 g. autoclaved 6 hr. at 145° in 3*N* HCl and the hydrolysate brought to 10 ml.) showed no trace of colour as compared with a control which contained the same reagents but which had not been heated. As an obvious colour was developed from 2 ml. standard solution containing 0·08 mg. hydroxyproline (B.D.H.), which is the smallest amount that could have been present in the test aliquots, assuming only 1 mol. hydroxyproline per mol. protein (mol. wt. 35,000), it can safely be concluded that this amino-acid is completely absent from Bence-Jones protein. This was confirmed later on the filtrate from the proline estimation.

Proline

The method of Engeland & Bastian [1937], which involves methylation of the mixed amino-acids and the isolation of dimethylproline by successive precipitation with $HgCl_2$, H_2PtCl_6 and $HAuCl_4$, has been used by Bastian [1937] who records a figure of 25–26·5 % for the proline content of gelatin as compared with the accepted figure of 19·7 % [Bergmann, 1935]. Guest [1939] has recently made use of the well-known condensation between oxidized proline and *p*-dimethylaminobenzaldehyde in acid solution in a method in which the oxidation is

effected by means of a suspension of PbO_2 in a phosphate buffer *p*H 8·7. As this procedure is stated to yield a satisfactory value for the proline content of casein and depends on there being little or no hydroxyproline present, a considerable number of tests and modifications, which need not be detailed, were made, but the method was finally abandoned. Consistent results could not be obtained with either pure proline or hydrolysates of casein or Bence-Jones protein. Moreover, in preliminary experiments with casein, the colours developed after acid hydrolysis (HCl and H_2SO_4) were so weak compared with those of baryta hydrolysates as to make the method suspect. The possibility was therefore examined of precipitating the proline on a small scale by means of ammonium rhodanilate [Bergmann, 1935].

In the large scale precipitation of proline, Bergmann has used various reagents including flavianic acid [Bergmann, 1935], phosphotungstic acid [Bergmann & Niemann, 1936] and alcohol [Bergmann & Niemann, 1937], mainly to remove arginine from the hydrolysate before addition of ammonium rhodanilate. Using aliquots from the same hydrolysate equivalent to 1·6–5·0 g. gelatin (Coignet Gold Label) the addition of flavianic acid was found to be unsatisfactory since it is difficult to remove excess from the precipitate of proline rhodanilate by washing on the filter; on the other hand, phosphotungstic acid results in a much cleaner solution and the amount added can be varied within fairly wide limits without affecting the result. As the final precipitate may easily be contaminated with excess of the ammonium rhodanilate which is not very soluble in water, it is necessary to have a fairly accurate idea of the amount of proline present, which may entail several trials, and to adhere to a fixed ratio between the volume of hydrolysate and that of the reagent. The correction factor for the solubility of proline rhodanilate as determined by Bergmann [1935] can then be applied. The details of the method finally adopted are as follows.

About 0·5 g. Bence-Jones protein was refluxed for 24 hr. with 10 ml. 20 % HCl. The humin was removed by centrifuging and washed twice with cold water. The total solution was concentrated three times *in vacuo* to remove HCl, transferred quantitatively to a 15 ml. centrifuge tube and treated dropwise with 1·0–1·4 ml. 10 % phosphotungstic acid, mixing well with a fine rod. After standing in the ice chest for 2 hr. the mixture was centrifuged and the liquid poured off and concentrated *in vacuo* to a gum. The residue was taken up in 3 ml. water and the solution transferred by means of a small teat pipette to a wide bore graduated test tube and adjusted to 4 ml. A solution of 0·14 g. ammonium rhodanilate in 2·4 ml. methyl alcohol was placed in the flask and the amino-acid solution pipetted in slowly with shaking, the pipette and test tube being washed with 2–3 drops methyl alcohol. After 2 hr. on ice the mixture was filtered on a sintered glass crucible and the residue washed three times with ice-cold water and dried in a desiccator. 0·460 g. protein gave 0·1345 g. proline rhodanilate (M.P. 133° as required). According to Bergmann's [1935] finding, the solubility correction[1] for 6·6 ml. mother liquor (37 % methyl alcohol) is 0·0165 g., giving a total of 0·151 g. proline rhodanilate or $0{\cdot}19 \times 0{\cdot}151$ g. proline. This is equivalent to 6·24 % of the moisture- and ash-free protein.

In one of the determinations the filtrate from the proline precipitation, without washings, was treated with 1 g. ammonium reineckate + 2 drops pyridine and left for 2 hr. on ice as described by Bergmann [1935] for the isolation of hydroxyproline. The mixture was then filtered and the residue washed

[1] Bergmann [1935] precipitated the proline rhodanilate from a solution containing 20 g. in 150 ml. methyl alcohol by adding 250 ml. 0·5 *N* HCl and recovered 19 g., i.e. a solubility of 1 g. in 400 ml. 37 % methyl alcohol which he loosely applies as a +5 % correction.

well and decomposed on the filter in aqueous suspension by adding 2 ml. pyridine. The resulting filtrate was acidified with acetic acid, the precipitate removed by centrifuging and the solution then tested for hydroxyproline by Guest's [1939] method. No trace of colour was produced, thus confirming the previous results.

Summary and discussion

The values for the various constituents are recorded in Table 1, along with those found by other investigators.

Table 1. *Comparison of analyses of Bence-Jones protein*

All the values are calculated as percentage of the moisture- and ash-free protein unless otherwise stated.

Constituent	Hopkins & Savory*	Abderhalden & Rostoski [1905]	Calvery & Freyberg†	Present author	Miscellaneous‡ 3	4	5
Ash	Trace	—	1·2–5·1	0·4	—	—	—
Moisture	Nil	—	4·8–10·6	7·5	—	—	—
Total N	16·2	—	18·1	14·7	14·8	16·07	16·01
Amino-N	—	—	14·3	—	—	—	—
Amide-N	1·3	—	1·0	1·0	—	—	—
Humin-N	—	—	Nil	0·14	—	—	—
Sulphur	1·18	—	1·0	1·34	—	—	1·24
Phosphorus	—	—	Nil	<0·01	<0·01	—	—
Tyrosine	4·20	1·7	6·7	8·8 (8·5)§	6·1	—	—
Tryptophan	0·82	—	2·5	1·4 (2·3)§	1·7	4·2	—
Cystine	0·57	—	3·0	2·9	—	—	—
Methionine	—	—	—	0·58	—	—	—
Arginine	6·04	—	5·1	3·6	—	—	—
Histidine	0·81	—	1·2	0·90	—	—	—
Lysine	3·67	—	6·8	4·0	—	—	—
Glycine	Present	1·7	—	—	—	—	—
Alanine	Present?	4·5	—	—	—	—	—
Leucine	6·51	10·6	—	—	—	—	—
Valine	5·60	—	—	—	—	—	—
Aspartic acid	2·12	4·5	4·6	—	—	—	—
Glutamic acid	7·8	6·0	8·6	—	—	—	—
Proline	2·70	1·9	—	6·3	—	—	—
Hydroxyproline	—	—	—	Nil	—	—	—
Phenylalanine	4·83	1·5	—	6·1	—	—	—

* The values of Hopkins & Savory [1911] are the mean of two consistent sets obtained from the protein of two separate patients.

† The values of Calvery & Freyberg [1935] are the mean for two specimens of protein excreted at different times by the same patient.

‡ 3, Hewitt [1929]; values uncorrected for ash. 4, Ohlsson & Nordh [1929]. 5, Magnus-Levy [1936].

§ Spectroscopic analysis.

It is of interest to consider these results firstly in relation to other evidence bearing on the nature of Bence-Jones protein. Physicochemical properties of the protein, including the refractive index, optical rotatory power and ultraviolet absorption spectrum have been investigated by Hewitt [1929]. The isoelectric point of different samples has been determined cataphoretically [Svedberg & Sjögren, 1929; Mainzer, 1932, 1, 2, 3; McFarlane, 1935; Magnus-Levy, 1936; Kekwick, 1940] as well as by electrometric titration [Jervell, 1932] and flocculation [cf. Magnus-Levy, 1936]. Svedberg & Sjögren [1929] and Magnus-Levy [1936] have reported measurements of the molecular weight by means of the ultracentrifuge and further work along these lines has been reported by Kekwick [1940] and others. The results of these investigations confirm

the immunological findings of Bayne-Jones & Wilson [1922, 1, 2], Robinson [1927] and others that Bence-Jones protein is of variable composition and is distinct from normal plasma proteins.

The extension of this work to the determination of the chemical structure of the protein has received little attention. Apart from the investigations of Lüscher [1922], Medes *et al.* [1935] and Tsutsui [1935] on the N-distribution, amino-acid analyses have been reported in only three cases [Abderhalden & Rostoski, 1905; Hopkins & Savory, 1911; Calvery & Freyberg, 1935]. While there are not as yet sufficient trustworthy data on the composition of the protein to give any certain indication of its pathological origin, there are however several points of interest in comparing the various values. Medes *et al.* [1935] examined, by the Van Slyke method, six different samples of the protein. Five of these were shown to be chemically identical within the limits of the method, the analyses being in close agreement with that of Lüscher [1922], while the sixth was quite different. Lüscher's [1922] analysis was made on material originally examined by Hopkins & Savory [1911] who considered that despite the admittedly imperfect methods of isolation then available, both the proteins examined by them as obtained from different patients were identical but distinct from the sample analysed by Abderhalden & Rostoski [1905]. Comparison of the figures obtained more recently by Calvery & Freyberg [1935] with those of the present paper and with miscellaneous values shows several divergencies. My N value is low, but it is unlikely that the inconsistency can be explained wholly on the basis of impurity. Hopkins & Savory's [1911] value, which is in agreement with those of Ohlsson & Nordh [1929] and of Magnus-Levy [1936], was obtained on 'practically ash-free' material dried at 110° and hence it is not to be brought into conformity with the higher value of Calvery & Freyberg [1935], as these authors suppose, by 'failure to correct for the ash and moisture content of the protein'. The S value is somewhat higher than has been obtained before but checks well with the determination of the S distribution. The values for the amino-acids also suggest that we are dealing with a protein chemically distinct from that of Calvery & Freyberg [1935]. The results of chemical analysis are therefore in agreement with evidence from other sources that Bence-Jones protein is of variable composition and that it represents a group of distinct but possibly similar proteins. There is little doubt that some of the samples which have been examined were chemically identical.

The origin of the protein remains obscure. The studies of Folin & Denis [1914], Medes *et al.* [1935] and others have shown that it cannot be considered of exogenous origin. The very considerable amount excreted in some cases precludes the possibility of its being a metabolite of the tumour. It seems more reasonable to regard it as the result either of the partial breakdown of a normal protein of higher molecular weight or of some hindrance to the complete synthesis of such a protein. In this way certain allied pathological conditions with which Bence-Jones proteinuria is associated might give rise to different products according to the response to different 'toxins'. On the basis of a chemical similarity first noted by Lüscher [1922], Calvery & Freyberg suggested that Bence-Jones protein may be derived from normal serum globulin by the latter being split into three particles of equal size; this would account for the molecular weight relation and for the excretion of Bence-Jones protein as a 'foreign' protein. This idea appears to entail the assumption that the protein is a single chemical entity of fixed composition. In view of the evidence to the contrary, the alternative suggestion demands consideration, viz. that the protein is the result of a deranged synthetic mechanism which would otherwise produce normal

plasma or other proteins. This would account more easily for the production of a protein which may vary not only in chemical composition and behaviour but also in mol. wt. It is interesting to note in this connexion that according to Bonsdorff *et al.* [1938] the mol. wt. of Bence-Jones protein determined on only a few occasions as 35,000 has once given a value of 70,000 while the myeloma protein discussed by them had a mol. wt. of about 200,000, which is higher than for any of the recognized plasma proteins.

Brief mention should be made of Meyler's [1936] recent experiments on the nature of the protein from bone marrow and leucocytes which suggest that small undetected amounts of Bence-Jones (or similar) protein normally derive from these sources, but that in myelomatosis and certain other conditions the output is so magnified above the rate of destruction in the body that the protein appears in the blood and is excreted as a pathological constituent. Confirmation and further work along these lines is desirable.

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